JOURNAL OF MASS SPECTROMETRY, VOL. 31, 1141–1148 (1996)

Effect of Alkyl Substitution at the Amide Nitrogen on Amide Bond Cleavage: Electrospray Ionization/Surface-induced Dissociation Fragmentation of Substance P and Two Alkylated Analogs

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Doubly protonated substance P and two analogs alkylated at the ninth position was studied to determine the effect of N-alkylation of the amide nitrogen on the electrospray ionization/surface-induced dissociation (ESI/SID) fragmentation pattern. Thermal decomposition experiments and *ab initio* calculations were also used in conjunction with the ESI/SID experiments. The increase in relative abundances of the product ions resulting from the cleavage of the amide bond at the alkylation site (relative to the corresponding cleavage for substance P) can be explained by the increased basicity of the amide nitrogen in the context of the 'mobile proton' model. The relative abundances of singly charged b ions suggest a rearrangement of the amide hydrogen located N-terminal to the bond cleaved.

KEYWORDS: surface-induced dissociation; peptide fragmentation mechanisms; amide N-alkylation; thermal dissociation; *Ab initio* calculations

INTRODUCTION

Dissociation of protonated peptides by tandem mass spectrometry has been explained to an extent by the 'mobile proton model', which relates the efficiency of peptide fragmentation to the ease of proton transfer along the peptide backbone.[1–21] The mobile proton model has evolved based on the observations of many researchers.[1–21] It argues that a proton that initially resides at a relatively basic site is transferred to less basic sites upon activation of the protonated peptide. In addition, the mobile proton model concludes that a large proportion of product ions formed during the dissociation of peptides by low-energy activation techniques [e.g. low-energy gas-phase collision-induced dissociation (CID)[22] and surface-induced dissociation (SID)[23]], result from charge-directed cleavage. According to *ab initio* and MNDO bond order calculations,[16,24,25] protonation at the amide nitrogen leads to a significant decrease in the amide bond order relative to that of the neutral unprotonated peptide (by about 35%), thus amide nitrogen protonated forms are hypothesized as plausible fragmenting structures for some charge-directed cleavages.

In the case of singly charged peptides, the electrospray ionization/surface-induced dissociation (ESI/SID) fragmentation efficiency (% fragmentation) has been shown to be dependent on the basic residues present.[18–20,26] It requires relatively higher energy to induce dissociation for a peptide that contains arginine or lysine than for ones without basic residues. Furthermore, it takes higher energy to dissociate a peptide containing an arginine than one containing a lysine residue. In the case of multiply charged peptides, however, one could expect protons of varying 'mobility', depending on the number of protons relative to the number of accessible basic residues, which could result in more efficient fragmentation than for the singly charged counterparts. Examples of easier fragmentation of doubly protonated peptides compared with their singly protonated counterparts have been reported in recent papers from our laboratory[19,27] and many such examples have been reported by other research groups.[3,4,7,10] As noted by many workers in the field, the ability of electrospray ionization[28–30] to produce multiply protonated peptides improves the analytical utility of tandem mass spectrometry for characterizing peptides.

As mentioned above, based on *ab initio* and MNDO bond order calculations,[16,24,25] it has been suggested that proton transfer to the amide nitrogen is critical to the charge-directed dissociation of the amide bond. A typical amide nitrogen is relatively less basic than the other sites in the peptide backbone that can be protonated, such as the N-terminal amine, carbonyl oxygens or basic side-chains. The energy difference between the most stable protonated form and that of the fragmenting, amide-protonated form should influence the ease

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CCC 1076–5174/96/101141–08

Received 11 April 1996
Accepted 26 June 1996

of fragmentation if charge-directed cleavage is involved. A peptide modification that alters the basicity of the amide nitrogen could be used to obtain experimental evidence for involvement of amide N-protonated forms. The basicity (and nucleophilicity) of the amide nitrogen should be increased by alkylation of the amide nitrogen. Such an N-alkylated amide bond would then be predicted to be more favorable for charge-directed cleavage than the less basic non-alkylated amide bond site. Two amino acids that contain alkylated amide nitrogen are proline (Pro) and sarcosine (Sar). In this work, we investigated the ESI/SID spectra of singly and doubly protonated (i) substance P (RPKPQQFF-**Gly**-LM-NH_2), (ii) Sar^9-substance P (RPKPQQFF-**Sar**-LM-NH_2) and (iii) Pro^9-substance P (RPKPQQFF-**Pro**-LM-NH_2) to monitor the influence of amide N-alkylation on the ESI/SID fragmentation of these peptides. Thermal decomposition, an alternative method of ion excitation and fragmentation, was also studied for the above peptides and the data were compared with the ESI/SID fragmentation pattern.

EXPERIMENTAL

Peptides were obtained from Sigma (St Louis, MO, USA) and were used without further purification (substance P, Sar^9-substance P, Pro^9-substance P). Samples for analysis were prepared by dissolving peptides in methanol–1% acetic acid solution (60:40), diluting to make ~50 μM solutions. The protonated peptides are generated with an electrospray ionization source. The electrospray source used in these studies is a modified version of the designs by Chowdhury *et al.*[31] and Papac *et al.*[32] The analyte solution was sprayed at atmospheric pressure from a syringe needle held at 4 kV into a heated metal capillary held at 120 V. Desolvation and thermal dissociation of ions occurred in the heated metal capillary, the temperature of which was measured at its outer wall. Thermal dissociation of the peptides was observed in the region of 250–360 °C and presumably occurred at the high pressure limit.[26,33] The desolvated ions were made to pass through a skimmer cone (90 V) and a set of focusing lenses before being introduced into the quadrupole mass analyzer. Experience shows that at a capillary–skimmer voltage difference of 30 V, gas-phase collisional activation in the capillary-skimmer region does not deposit a significant amount of internal energy to the selected projectiles (e.g. even small non-basic di-or tripeptides such as G-G, AA, G-Sar, GGG and AAA do not fragment in the ion source under these conditions). Note that for the thermal decomposition experiments the capillary–skimmer potential difference was kept at 5 V.

A custom tandem quadrupole mass spectrometer for low-energy ion–surface collisions was used to perform these experiments.[34] The tandem mass spectrometer consisted of two 4000 u Extrel quadrupoles arranged in a 90° geometry with the surface positioned at the intersection of the ion optical paths of the quadrupoles. For the ESI/SID studies, the precursor ions of interest were mass selected by the first quadrupole and made to collide with the surface. The fragment ions were analyzed by the second quadrupole. The collision energy of the incident ions was determined by the potential difference between the skimmer and the surface, multiplied by the charge state of the precursor ion selected. A chemically modified surface, a self-assembled monolayer of 2-(perfluorooctyl)ethanethiol on gold, was used in these experiments. The preparation of the fluorinated self-assembled monolayer is described in more detail elsewhere.[35] For thermal decomposition, no ion selection was made and no surface collisions were applied.

Ab initio calculations at the MP4SDTQ 6–31G**//HF6–31G** level were performed by using the program package Gaussian 94.[36a] Bond orders were calculated according to the Mulliken–Mayer formalism.[36b–d]

RESULTS AND DISCUSSION

ESI/SID spectra obtained by 55 eV collisions of doubly charged substance P, Sar^9-substance P and Pro^9-substance P with the fluorinated monolayer surface are shown in Fig. 1(a), (b) and (c), respectively. A general

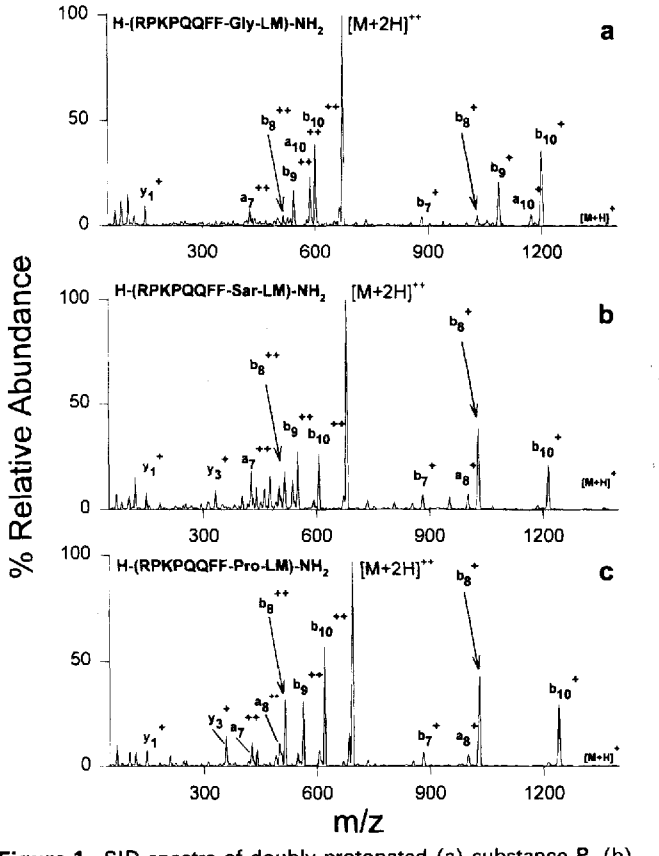


Figure 1. SID spectra of doubly protonated (a) substance P, (b) Sar^9-substance P and (c) Pro^9-substance P. The doubly protonated precursor ions were generated by ESI. All SID spectra were obtained by 55 eV collisions of the selected doubly protonated peptides with a 2-(perfluorooctyl)ethanethiolate self-assembled monolayer surface.

feature of the ESI/SID spectra of the three peptides is that singly and doubly charged **b** (and corresponding **a**) type ions are the dominant product ions not only at 55 eV but over most of the collision energy range studied (30–80 eV). However, differences are observed in the spectra of the alkylated analogs in comparison with that of substance P in the relative ratios for both singly and doubly charged product ions [compare Fig. 1(a) with Fig. 1(b) and (c)]. The most prominent differences are the enhanced abundances of the singly and doubly charged $\mathbf{b}_8$ and $\mathbf{a}_8$ type ions (marked by arrows) and the absence of the singly charged $\mathbf{b}_9^+$ and $\mathbf{a}_9^+$ ions in the spectra of the alkylated analogs. In addition, the abundance of the singly charged $\mathbf{y}_3^+$ ion (which could be a complementary ion formed when $\mathbf{b}_8^+$ is formed from $[M + 2H]^{2+}$) increases for the alkylated analogs relative to substance P.

To illustrate the relative differences in the abundances of the characteristic singly and doubly charged $\mathbf{b}_8$–$\mathbf{b}_{10}$ ions as a function of SID collision energy, energy-resolved mass spectra (ERMS) are also presented. The abundances of the corresponding $\mathbf{a}_8$–$\mathbf{a}_{10}$ ions, presumably formed by CO loss from the **b** ions, are added to the **b** ion abundances. Figures 2 and 3 show the relative abundances of doubly and singly charged $\mathbf{b}_8 + \mathbf{a}_8$, $\mathbf{b}_9 + \mathbf{a}_9$ and $\mathbf{b}_{10} + \mathbf{a}_{10}$ ions, respectively, for the three peptides, studied over the SID collision energy range 30–80 eV. For the alkylated analogs, the increase in the abundance of both singly and doubly charged $\mathbf{b}_8$ (and $\mathbf{a}_8$)product ions is clear from the spectra in Fig. 1 and the ERMS curves in Figs 2 and 3 (filled squares), as is the absence of $\mathbf{b}_9^+$ and $\mathbf{a}_9^+$ (open triangles).

Thermal decompositions of substance P and the alkylated analogs were also investigated in the heated

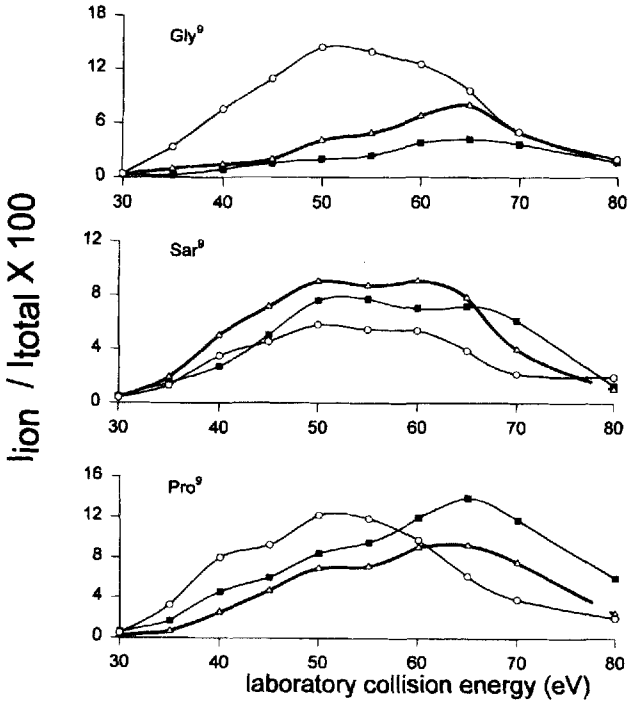


Figure 2. Plots of ion abundance *vs.* SID collision energy for the formation of ion types $\mathbf{b}_8^{2+}$ and $\mathbf{a}_8^{2+}$ (filled squares), $\mathbf{b}_9^{2+}$ and $\mathbf{a}_9^{2+}$ (open triangles) and $\mathbf{b}_{10}^{2+}$ and $\mathbf{a}_{10}^{2+}$ (open circles) for (a) substance P, (b) Sar⁹-substance P and (c) Pro⁹-substance P.

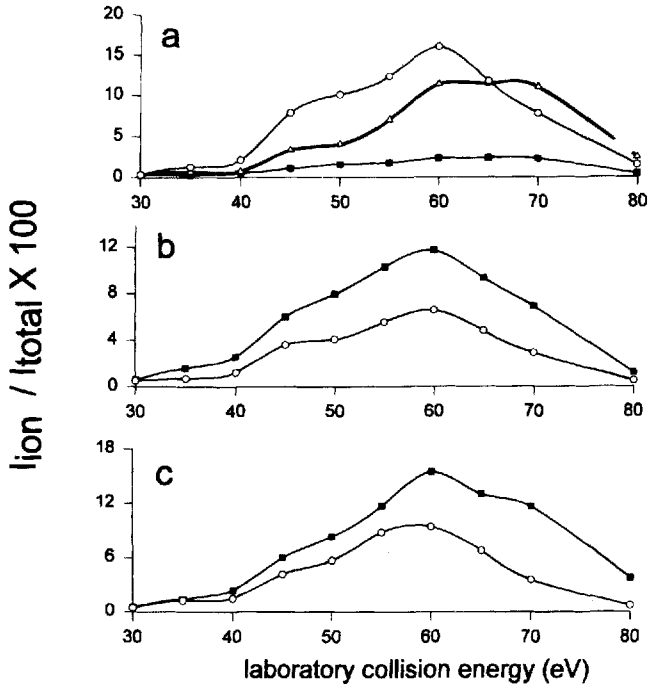


Figure 3. Plots of ion abundance *vs.* SID collision energy for the formation of ion types $\mathbf{b}_8^+$ and $\mathbf{a}_8^+$ (filled squares), $\mathbf{b}_9^+$ and $\mathbf{a}_9^+$ (open triangles) and $\mathbf{b}_{10}^+$ and $\mathbf{a}_{10}^+$ (open circles) for (a) substance P, (b) Sar⁹-substance P and (c) Pro⁹-substance P.

capillary of the ESI source. In the present case, we did not use an external heated capillary as was described in our recent paper.[33] Although ion selection prior to thermal activation cannot be achieved by using this set-up, the general pattern of the thermal decomposition spectra may provide information on fragmentation pathways associated with lower internal energies and different time-scales (ms) in comparison with SID (ms). Figure 4 shows the thermal decomposition spectra of Sar⁹-substance P obtained at 300, 340 and 360 °C. The relative order of ion formation is reflected by these spectra. At lower temperatures, in the region of about 250–320 °C, only doubly charged $\mathbf{b}_{10}^{2+}$, $\mathbf{b}_9^{2+}$ and $\mathbf{b}_8^{2+}$ fragments are observed [see Fig. 4(a)]. These processes are followed by the formation of the singly charged $\mathbf{b}_8^+$ ions [see Fig. 4(b) at 340 °C]. At this temperature, some of the $\mathbf{b}_n^{2+}$ ions fragment further to the corresponding $\mathbf{a}_n^{2+}$ ions. At slightly higher temperatures [Fig. 4(c), 360 °C], some of the $\mathbf{b}_8^+$ ions fragment further to $\mathbf{a}_8^+$ ions. It is important to note that both doubly charged $\mathbf{b}_8^{2+}$ and singly charged $\mathbf{b}_8^+$ ions are absent in the thermal decomposition spectra of substance P obtained at the same temperatures as shown in Fig. 4. The absence of these ions is in agreement with the ESI/SID observations and indicate clearly the influence of the N-alkylation at the ninth position (see below).

Fragmentation efficiency data (% fragmentation) show that the fragmentation of mass-selected singly protonated substance P and Sar⁹/Pro⁹ analogs requires much higher SID collision energies than are required for the fragmentation of their doubly protonated counterparts. It has been observed in previous work in our laboratory[37] and elsewhere [38] that the fragmentation efficiency of singly charged substance P improves

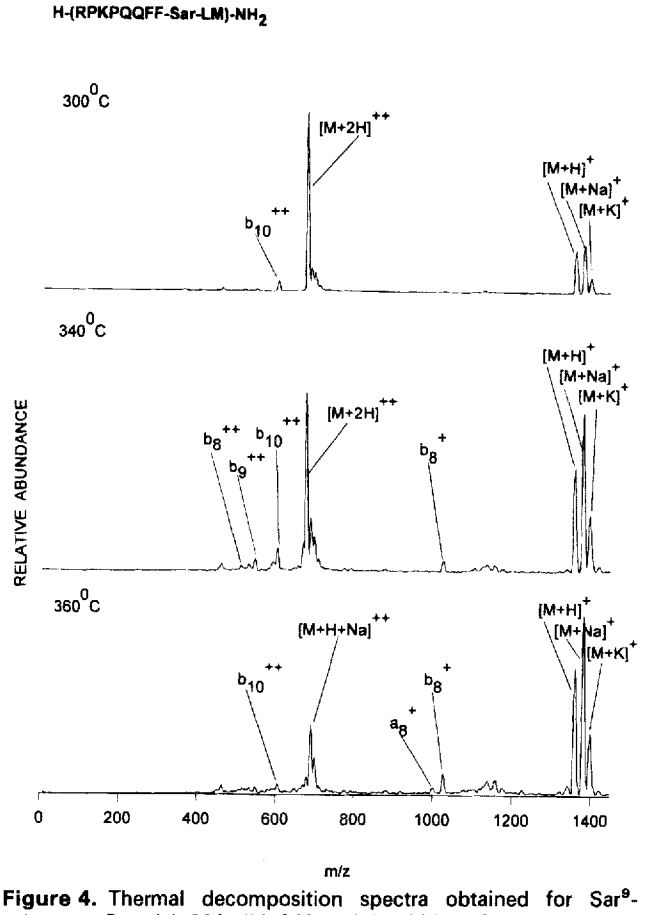


Figure 4. Thermal decomposition spectra obtained for Sar^9-substance P at (a) 300, (b) 340 and (c) 360 °C. Thermal decomposition occurs in the heated capillary of the electrospray source.

drastically when the arginine residue is removed. Based on other data from our laboratory,[19] this indicates that in singly protonated substance P, the proton is located at the basic arginine side-chain and the removal of this residue makes the proton more 'mobile', inducing efficient fragmentation at low collision energies. (Note that "localization" of the proton at a basic side chain does not preclude additional Solvation of the proton by other heteroatoms in the peptide.) In comparison with other data from our laboratory, the doubly protonated substance P and Sar^9/Pro^9 analogs have dissociation onsets lower than doubly protonated peptides containing two arginine residues but equivalent to or only slightly higher than the doubly protonated peptides containing a single available arginine.[21] The energetics of fragmentation of doubly protonated substance P and the Sar^9/Pro^9 analogs thus suggest that a 'mobile' proton is instrumental in inducing the relatively efficient fragmentation observed (leading to both singly and doubly charged product ions).

Because a major premise of this work is that alkylation of the amide nitrogen will have an influence on peptide dissociation patterns, *ab initio* quantum chemical calculations were also performed to evaluate the relative order of proton affinities for simple model compounds: acetamide, N-methylacetamide and N,N-dimethylacetamide.The relevant results from these calculations are presented in Table 1. As expected, energetic calculations at the MP4SDTQ 6–31G**//HF 6–31G** level predict that the proton affinity values increase in the order acetamide < N-methylacetamide < N,N-dimethylacetamide (note that the proton affinity values for the amide nitrogen are similar to those reported recently by Uggerud and co-workers for glycinamide[39]). The influence of protonation of the amide nitrogen on amide bond lengths and amide bond orders was also evaluated for these simple model compounds. In agreement with earlier predictions published for simple model peptides by both *ab initio* and MNDO methods,[16,24,25] protonation on the amide nitrogen of acetamide analogs leads to a significant weakening of the amide bond, indicating its easy cleavage in amide nitrogen-protonated forms. It is worth noting that the calculated bond orders shown in Table 1 for these simple acetamide derivatives are very close, even in their absolute values, to those published for other model peptides, such as di-and triglycines and dialanines.[16,24,25] This indicates that the weakening of the amide bond is essentially due to a 'local' effect of protonation at the amide nitrogen and practically independent of the position of the amide bond (for the effect of hydrogen bonds, see Ref. 16).

Table 1. Proton affinities, amide bond lengths and amide bond orders of acetamide and its alkylated analogs and their protonated forms

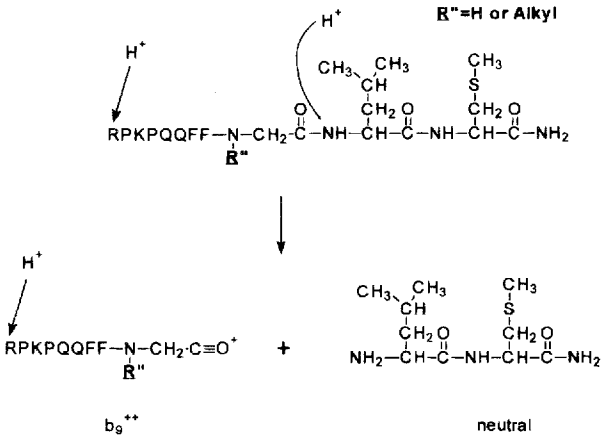
Species	Proton affinity (kJ mol)[a]	C—N bond length (pm)	C–N bond order[b]
CH_3CONH_2	830.9	135.6	1.047
$CH_3CONH_3^+$		155.5	0.651
$CH_3CONHCH_3$	862.3	135.7	1.059
$CH_3CONH_2CH_3^+$		153.1	0.691
$CH_3CON(CH_3)_2$	885.8	136.2	1.052
$CH_3CONH(CH_3)_2^+$		152.2	0.701

[a] Proton affinities were calculated from MP4SDTQ 6–31G**// HF6–31G** total energies corrected by 0.9 × ZPVE (zero-point vibrational energy) values.
[b] Bond orders were calculated according to the Mulliken–Mayer formalism.[36b–d]

Because both the ESI/SID and thermal dissociation experiments show significant differences in the fragmentation pattern between substance P and the Sar^9/Pro^9 analogs, it is reasonable to assume that alkylation of the amide-N of the ninth residue plays a role in the fragmentation mechanisms. To explain the ESI/SID experimental data presented above, both charge-directed cleavages, which are initiated at the charge site, and charge-remote cleavages, which occur remote to the site of charge, should be considered as possible fragmentation mechanisms.

Formation of b_n^{2+}/a_n^{2+} ions

First consider the enhanced abundances of b_8^{2+} and a_8^{2+} doubly charged fragment ions in the ESI/SID spectra of the Sar^9- and Pro^9-substance P derivatives [Fig. 1(b) and (c) relative to those for substance P (Fig. 1(a)]. One plausible explanation for the enhanced abundances of



Scheme 1

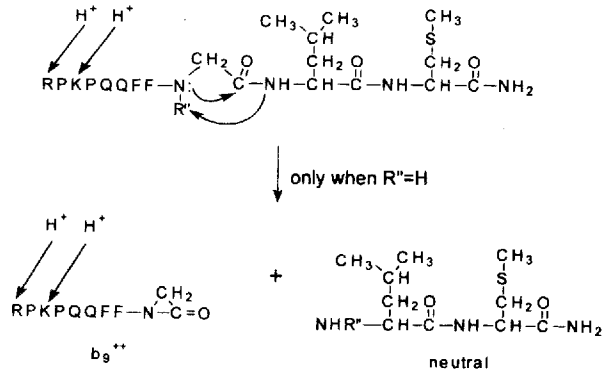
b_8^{2+}/a_8^{2+}, which are also observed in the thermal decomposition spectra at lower temperatures, is a charge-directed process, amide-N protonation with subsequent direct formation of doubly charged **b** ions (Scheme 1). Obviously, the other doubly charged **b** ions can also be formed by charge-directed simple bond cleavages: activation would lead to fragmenting structures in which one proton may be located on arginine with the other 'mobile' proton located on the appropriate amide nitrogen. Although the relative basicities of the various sites in a typical peptide indicate that the amide-N should be one of the least preferred sites for protonation,[24,25,39] activation of the doubly protonated peptide (e.g. by collisions with a gaseous target or surface) can promote proton transfer to amide-N. (Note that we use the term 'mobile proton' not only for proton transfers from the side-chain to the backbone but also to describe the local mobility such as proton transfer from amide-O to amide-N of the same amide bond.) Alkylation of the amide-N, as in Sar^9/Pro^9-substance P, should enhance the probability of proton transfer to this position, leading to enhanced cleavage consistent with the abundant b_8^{2+}/a_8^{2+}. This is supported by the *ab initio* proton affinity data in Table 1.

To explain the data presented above, we must account not only for enhanced cleavage at the ninth (alkylated) residue but also for the enhanced abundances of the larger **b** ions (8–10) compared with the smaller $\mathbf{b}_n$ ions ($n < 7$). If activation promotes proton transfer from more basic to less basic sites, then a population of different protonated forms should be produced. The distribution of various 'activated' forms (one proton on arginine and the other proton moved to less basic sites) should depend on the relative energies of the different forms but they are not necessarily in thermodynamic equilibrium. For example, it has been shown by Wesdemiotis and co-workers[40] that the most basic site of glycine is not exclusively protonated under kinetically controlled conditions.

Some of the various protonated forms may not be fragmenting structures, e.g. amide O-protonated forms may exist in higher proportions than amide N-protonated forms, but the bond order calculations suggest that the amide N-protonated forms are the ones most likely to fragment readily without further rearrangement of the protons.[16,24] The calculated bond orders in the amide N-protonated N-methyl-and N,N-dimethylacetamide are very close to each other. This suggests that the critical energy of the charge-directed cleavage of the amide bond in glycine and sarcosine derivatives should not differ significantly. In other words, the charge-directed formation of the doubly charged **b** ions for the peptides investigated can be considered as a two-step process which seems to depend mainly on the relative energetics of the proton transfer (the formation of the population of different protonated forms) and not on the energetics of the amide bond cleavage. (To confirm this hypothesis further, transition-state calculations would be required, but this is beyond the scope of this paper.)

Although the relative energies of the different protonated forms should be dependent on the relative basicities of individual protonation sites, the formation of the overall population of different protonated forms could depend on additional factors such as Columbic repulsion or secondary structure effects. If cleavage indeed occurs by charge-directed fragmentation as indicated in Scheme 1, the abundant b_8^{2+}/a_8^{2+}, b_9^{2+}/a_9^{2+} and b_{10}^{2+}/a_{10}^{2+} ions imply that those forms in which one of the last three residues is protonated are more abundant. It is difficult to conceive a charge-remote mechanism that would lead to enhanced formation of high m/z members of the **b** ion series over low m/z members, although it is possible that a charge-remote fragmentation mechanism could lead to enhancement of cleavage at the alkylated residue.

One possible charge-remote mechanism for doubly charged **b** ion formation is shown in Scheme 2. This mechanism is similar to that drawn for the formation of singly charged **y** ions from singly protonated peptides by several workers based, e.g., on H–D exchange studies[13,15] and neutralization–reionization experiments.[41] A similar mechanism (involving the amide hydrogen) has also been suggested by Boyd and co-workers[10] and more recently by Adams *et al.*[42] for doubly protonated peptides. As suggested by previous ESI/SID experimental observations,[18–20] prior to surface collision the protons are expected to be located at the most basic sites which are the N-terminal arginine and the lysine in substance P and its Sar^9/Pro^9 alkylated derivatives (see Scheme 2). It is conceivable that cleavage of the amide bond by this mechanism

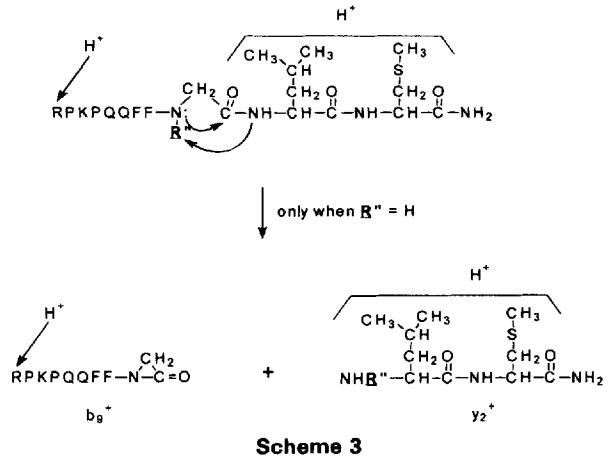


Scheme 2

might be enhanced if alkylation at the amide nitrogen increases the likelihood of the rearrangement reaction, perhaps by increasing the nucleophilicity of the amide nitrogen. According to this mechanism, the doubly charged $\mathbf{b}_9^{2+}$ ions should be absent in the ESI/SID spectra of the alkylated analogs because the amide hydrogen that is involved in this mechanism is not available at the ninth position in the alkylated derivatives and the spectra indicate no alkyl group transfer. However, the $\mathbf{b}_9^{2+}$ ions are present with considerable abundance in the ESI/SID spectra of the alkylated derivatives. The charge-remote mechanism sketched in Scheme 2 therefore cannot be the major fragmentation pathway for the formation of doubly charged ions. (A related mechanism involving transfer of hydrogen from the carbon backbone could also be considered but would not explain why the spectra change dramatically upon amide N-alkylation.) The fact that the abundance of doubly and singly charged $\mathbf{b}_n$ ions with lower mass ($n < 7$) is of much lower abundance than those of higher mass $\mathbf{b}_n$ ions ($n = 7$–10) further suggests that the mobile proton is involved in their formation. If protons remain localized at the arginine and lysine as expected based on the relative basicities of various sites available for protonation, there would be no reason to expect enhanced abundances at positions 9–11, i.e. $\mathbf{b}_7$, $\mathbf{b}_6$, $\mathbf{b}_5$, etc. should be as prominent as $\mathbf{b}_9$ and $\mathbf{b}_{10}$ in the spectra of substance P and $\mathbf{b}_8$ and $\mathbf{b}_{10}$ in the spectra of the alkylated derivatives.

Formation of $\mathbf{b}_n^+/\mathbf{a}_n^+$ ions

While it is straightforward to rationalize the formation of the doubly charged **b** ions by a charged-directed heterolytic bond cleavage (Scheme 1), it is difficult to envision the formation of singly charged $\mathbf{b}^+$ ions in the SID spectra of doubly protonated substance P and Sar^9/Pro^9 substance P by a similar mechanism. Based on the absence of $\mathbf{b}_9^+/\mathbf{a}_9^+$ in the spectra of the doubly protonated alkylated derivatives, it may be inferred that the amide-H located N-terminal to the site of cleavage may be involved in the formation of the singly charged $\mathbf{b}_n^+$ ion series. A 1,4-H rearrangement (Scheme 3, which is similar to that shown in Scheme 2) is a logical mechanism leading to the formation of the singly charged $\mathbf{b}_n^+$ ions: the absence of $\mathbf{b}_9^+/\mathbf{a}_9^+$ ions in the respective spectra is consistent with the lack of the corresponding amide hydrogens available at alkylated amide nitrogen. (Note that, in principle, the absence of $\mathbf{b}_4/\mathbf{a}_4$ ions in the spectra of substance P and the alkylated derivatives could also be related to the lack of amide H but because the lower $\mathbf{b}_n$ ions ($n < 7$) are of very low abundance it is not highlighted here.)



Scheme 3

The suggested mechanism leading to the formation of singly charged **b** and **y** ions is the same as that proposed by Adams et al.[42] for the formation of $\mathbf{b}_6^+$ and $\mathbf{y}_2^+$ by high-energy CID for angiotensin II (DRVYIHPF). Angiotensin II is similar to the peptides investigated in the present study in that it has Arg near the N-terminus and a Pro in the backbone. It is possible that alkylation of the amide nitrogen may play a role in determining the rate of this rearrangement process which could induce enhanced cleavage at the alkylated position leading to more abundant $\mathbf{b}_8^+/\mathbf{a}_8^+$ ions as observed in the ESI/SID spectra of the alkylated derivatives. Such a mechanism is further supported by the observation of $\mathbf{y}_3^+$ ions for the alkylated derivatives. The relative ratios of $\mathbf{b}_8^+$ to $\mathbf{y}_3^+$ ions in the ESI/SID spectra indicate that the formation of $\mathbf{y}_3^+$ may not be completely complementary to the formation of $\mathbf{b}_8^+$ ions because the relative abundances of these ions plotted as a function of SID collision energy have different slopes. This suggests that a 1,4-rearrangement may not be the only process leading to the formation of $\mathbf{b}_n^+$ ions or that the $\mathbf{y}^+$ ions may further dissociate to internal fragments.[2] (A comparison with the $\mathbf{b}_6^+/\mathbf{y}_2^+$ ion formation from doubly charged angiotensin II by ESI/SID in our instrument suggests that the different slopes for the $\mathbf{b}_8^+/\mathbf{y}_3^+$ ions is not the result of an ion collection problem.) As an alternative mechanism, one may consider the mechanism suggested recently by Harrison and co-workers[43] for the formation of singly charged $\mathbf{b}_2^+$ ions from singly protonated peptides. According to their mechanism, which involves a nucleophilic attack of carbonyl oxygen on the carbon of another carbonyl group, the absence of $\mathbf{b}_9^+$ ions in our spectra would not be expected. The almost complete absence of the $\mathbf{b}_9^+$ ions indicates that the H transfer may be the key process in any mechanism that may be operative apart from that proposed in the present paper (Scheme 3). At this point it is not clear whether the formation of the singly charged **b** ions should be categorized as 'charge-remote' or 'charge-directed;' the fact that only high-mass singly charged **b** ions are formed and that the ions form readily from doubly protonated precursors suggests some involvement of the proton.

CONCLUSIONS

In accordance with work published[7,10,14,19] and in progress,[21,26] the analysis of the ESI/SID spectra of doubly charged substance P and its Sar^9- and Pro^9-analogs discussed in this paper further confirms that ESI/SID can provide useful analytical information regarding the sequence of peptides and also provides important mechanistic information on the gas-phase fragmentation of protonated peptides. The ESI/SID

results reported here show that alkylation at the amide nitrogen plays a distinct role in fragmentation. These results are related to the observations of extensive cleavage at proline residues reported by many researchers.[1a,44–46] Based on the relative abundance of singly and doubly charged fragment ions of doubly protonated substance P and two alkylated analogs in the ESI/SID and thermal decomposition spectra, we conclude that doubly charged $\mathbf{b_n^{2+}}$ ions are mainly formed by a low-energy, charge-directed simple bond cleavage of the amide bond triggered by proton transfer to the amide nitrogen. On the other hand, the singly charged $\mathbf{b_n^{+}}$ ions are formed via a rearrangement process in which the amide hydrogen plays an essential role. In general, however, neither of these mechanisms can be regarded as exclusive: more than one mechanisms may be operative for both doubly and singly charged product ion formation. To reveal further details of these mechanisms, additional efforts must be made, including, e.g., H–D exchange experiments, D labelling, high mass resolution and extended time-scale of fragmentation.

Acknowledgements

This work was stimulated, in part, by questions asked at the 21st meeting of the British Mass Spectrometry Society and discussions with Professor Simon Gaskell, Mark Bolgar and other group members. The work was funded by a grant from NIH (grant No. RO1 GM51387).

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