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ScienceDirect
International Journal of Mass Spectrometry 267 (2007) 183–189
International Journal of Mass Spectrometry
www.elsevier.com/locate/ijms

Generation of di-lithiated peptide ions from multiply protonated peptides via ion/ion reactions

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Received 15 November 2006; received in revised form 7 February 2007; accepted 20 February 2007
Available online 23 February 2007

Abstract

Gas phase conversion of multiply protonated peptides to alkali di-metalated peptide ions via ion/ion reactions is demonstrated as an alternative strategy to forming alkali di-metalated ions from solution. Previously, alkali di-metalated peptide ions have been generated by direct electrospray (ESI) of solutions that contain both peptides and a salt of the metal of interest. Here, di-lithiated peptides are generated in the gas-phase via ion/ion reactions of multiply charged protonated peptides with metal containing anions ($Metal_2L_3^-$) where lithium is the metal, and L is a singly charged anionic ligand. Fragmentation spectra of a series of di-lithiated model peptide ions $[Pep\text{-}H + 2Metal]^+$ formed via ion/ion reactions are compared with fragmentation of ions of the same molecular formula formed directly from nano-electrospray ionization (nano-ESI) of solutions that contain metal salts and peptides. Di-lithiated ions formed in the gas phase fragment in a similar manner to those formed directly from nano-ESI of a solution containing metal salt and peptide. Low energy beam type collisionally induced dissociation (CID) is also demonstrated as a tool to form the ion of interest from an intermediate complex that is formed during an ion/ion reaction.

Keywords: Ion/ion reaction; Gas phase ion synthesis; Di-lithiated peptide; MS/MS; Dual nano-electrospray ionization

1. Introduction

Gaseous protonated peptides play an important role in modern protein analysis both because they can provide primary sequence information upon activation [1] and because they can be readily formed by several ionization methods, such as fast atom bombardment (FAB) [2], matrix assisted laser desorption ionization (MALDI) [3–5], and electrospray ionization (ESI) [6]. Protonated peptides subjected to collisional activation frequently cleave at amide linkages to yield complementary b_n/y_n series fragment ions that can be used to extract primary sequence information [1]. However, side chain cleavages and losses of small molecules, such as water and ammonia, can also compete. Furthermore, products from only one or a few specific cleavages often dominate product ion spectra, depending upon the charge state and the primary sequence of the peptide.

Although fragmentation of protonated peptides has been demonstrated to be highly useful, the sequence information provided by protonated peptides can be limited due to the fact that the protonated peptides may not fragment at every amide bond, leading to incomplete sequence information. For this reason, alternative means for deriving peptide sequence information are desirable. One such alternative approach is to employ metalated peptide ions rather than protonated peptides as the surrogate for peptide sequencing [7–30]. Peptides complexed with a single alkali metal cation, for example, have been observed to undergo a unique fragmentation pathway, in which the C-terminal amino acid residue is cleaved off leaving the metal–peptide cation referred to as $[b_n + OH + Metal]^+$, where Metal = Li, Na, K [7–16,27–32]. This fragmentation pathway is not observed if the carboxylate group of the C-terminus is derivatized [11,15]. This process can be useful for sequencing peptides via an MS^n experiment that successively cleaves the C-terminal amino acid from the peptide ion and its intermediate products [27,30,33]. Two similar mechanisms have been proposed by Gronert and co-workers [34] and Farrugia and O'Hair [35], which posit the C-terminal residue loss as proceeding through a mixed anhydride intermediate. For singly alkali metalated-dipeptides, this mixed anhydride mechanism results in the loss of sequence information, because the mixed anhydride intermediate leads

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doi:10.1016/j.ijms.2007.02.030

to the same products regardless of the starting sequence. In two recent papers, Wesdemiotis and co-workers have shown that when two alkali metal cations are present in the metal–peptide complex $[Pep\text{-}H + 2Metal]^+$ (Metal = Li, Na) the problem of sequence information loss is avoided because fragmentation does not pass through the mixed anhydride intermediate. Instead, in the di-metalated peptide ion one alkali metal atom provides the charge, and another replaces the proton on the C-terminus (COOMetal) [36,37]. In this scenario, the conventional peptide nomenclature [1] must be modified to account for the attachment of metalated ions. Ions containing alkali metal atoms are depicted by adding (*) for one metal ion, and (**) for two metal atoms [36,37]. For example, a cleavage which breaks the amide bond and results in the ion containing the N-terminus and one metal ion would be denoted b_n^* while the same peptide fragment containing two metal atoms would be depicted by b_n^{**}.

The most common method of forming gas phase metalated ions has been to combine peptides and metal salts in one solution, and then forming ions via direct ionization of this mixture, either through FAB [36], ESI [5,27,30,37,38], or MALDI [3,4,37]. Electrospray ionization of peptide or protein containing solutions generally result in a distribution of charge states arising from the attachment of multiple cations. Direct electrospray of metal–peptide solutions can result in observation of metal-cationized ions, but there are several disadvantages to this method. Depending on the ionization and solution conditions, the ions observed in the mass spectrum will be comprised of a variety of different combinations of metal ions and protons. The presence and abundance of metal cationized peptides can be highly dependent upon solution composition and pH. The desired metal–peptide ion of interest may not be readily formed as the dominant species, and finding the optimum conditions may be time consuming. Electrospray ionization of solutions containing metal salts and peptide can also suffer from signal suppression, depending upon salt concentration [39].

Gas-phase ion/ion reactions offer an alternative approach for the formation and manipulation of metalated peptide ions from multiply protonated peptides through a reaction sequence referred to as 'cation switching' [40–43]. The ion/ion reaction approach provides a high degree of control over the identities of the reactant ions when they are performed within the context of an MS^n procedure. Using ion/ion reactions also allows the oppositely charged reactants to be ionized and optimized separately to achieve maximum signal for the ions of each polarity, thereby avoiding problems of signal suppression which may be observed if the metal and peptides are mixed in the condensed phase. Previous cation switching studies have emphasized the replacement of two or more protons in a multi-protonated peptide with a single mono-valent or multi-valent metal ion. In this study, we examine the transfer of two lithium metal ions into a doubly protonated peptide to form the $[Pep\text{-}H + 2Li]^+$ species of interest via a cation switching process. The dissociation behavior of the resulting di-lithiated peptide ions formed via ion/ion reactions is then compared with that observed for ions of the same molecular formula formed directly from nano-ESI of a mixture of salt and peptide in solution.

2. Experimental

2.1. Materials

Lithium chloride (Mallinckrodt, Phillipsburg, NJ) and lithium hydroxide (Sigma, St. Louis, MO) were obtained from commercial sources and used without further purification. Lithium chloride was used for all ion/ion reaction experiments. Salt solutions of lithium chloride were prepared for nano-electrospray (nano-ESI) by dissolving 1–2 mg/mL in acetonitrile. Peptides angiotensin II (DRVYIHPF) and bradykinin (RPPGFSPFR) were obtained from Sigma (St. Louis, MO), VDPVNFK and KGAILKGAILR were synthesized by SynPep (Dublin, CA), and YGGFLK was obtained from BACHEM (King of Prussia, PA). The NFFWK peptide was obtained from the enzymatic digestion of somatostatin-14 by trypsin, followed by HPLC separation. Peptide and protein solutions (20–50 μM) for nano-ESI were prepared in a (% by volume) solution of 49:49:2 methanol/water/acetic acid. Typical nanospray voltages were between 0.85 and 1.5 kV for peptides, and −1.0 and 2.5 kV for metal salts. For comparison of ions formed via ion/ion reactions with those formed directly from solution phase mixture of peptides and proteins, solutions containing 50 μM peptide and 5.0 mM alkali-metal salt of lithium chloride or lithium hydroxide were combined and subjected to nano-ESI.

2.2. Instrumentation

All experiments were performed using a prototype version of a QTRAP mass spectrometer [44] (Applied Biosystems/MDS Sciex, Concord, Ont., Canada) that has been modified for ion/ion reactions [45,46]. All experiments were controlled by a research version of the Daetalyst 3.10 software provided by MDS Sciex. The electronics of the QTRAP instrument were modified to allow superposition of an auxiliary radio frequency (rf) signal to the containment lenses of the second and third quadrupole arrays of the instrument. The frequency of the auxiliary rf signal was optimized for each ion/ion reaction experiment at a fixed 100 $V_{0\text{–}p}$. For ion/ion reaction experiments, the high-voltage power supply for the nanospray source of peptide ions was pulsed on while the first quadrupole (Q1), operated in rf/dc mode, was used to transmit the isolated ions of interest to the second quadrupole (Q2) where the ions were trapped. The ions were cooled in Q2, which was held at a pressure of 6–8 mTorr of nitrogen, for 50 ms, during which time the high voltage on the first emitter was turned off. After the cooling step, the power supply connected to the second nano-ESI emitter, which was operated in polarity opposite to that for the first nano-ESI emitter, was triggered on while the dc potentials applied to the ion path before Q2 were adjusted to allow ions of the opposite polarity to enter Q2. The positively and negatively charged ions were held together in Q2 over a variable reaction period. After the mutual storage time, any remaining reagent anions were removed by applying an attractive dc potential on the Q2 containment lenses while the auxiliary rf signals were terminated. The product ions from the cation switching ion/ion reaction were transferred into Q3 (3.5×10^{-5} Torr), where they were isolated using rf/dc isola-

tion before they were subjected to ion trap collisional activation. Fragment ions resulting from CID were subsequently subjected to mass-selective axial ejection [47] using a supplementary rf signal at frequency 380 kHz. In cases where beam type CID was applied to the products of ion/ion reaction as they were transferred from Q2 to Q3, the dc offset was typically between −12 and −16 V. The spectra shown are typically the averages of 10–100 individual scans.

3. Results and discussion

3.1. Cation switching to form di-lithiated peptide ions

Previous cation switching studies made use of a modified quadrupole ion trap instrument with multiple ion sources whereby reactant ion selection was effected by ejection of ions other than those of interest following ion accumulation [40–42]. A drawback to this approach is a compromised capability for selection of reactants in the second ion polarity population admitted into the ion trap. When a mixture of reactant ions is present, all those reactant species can react during the reactant ion accumulation period. Also, after reactant ion accumulation, care must be taken to avoid ejection of the first ion population when ejecting unwanted reactant ions. In some cases, it is not possible to avoid contributions from side reactions when the "fill first/eject later" approach to reactant selection is used. The present system overcomes this problem because ions of each polarity are mass selected by Q1 in conjunction with accumulation in Q2. This capability greatly improves the ability to define precisely the nature of the ion/ion reactants and is particularly useful when a mixture of ions is present, as is the case when salts are subjected to electrospray. It is a key advantage when it is desirable to be able to transfer a precise number of metal ions into a polypeptide ion.

Cation switching studies performed to date have emphasized the incorporation of a single metal ion into a polypeptide cation using negatively charged metal–ligand complexes. For the case of a doubly protonated peptide and a monovalent metal cation, the generic cation switching reaction is

$$[\mathrm{Pep} + 2\mathrm{H}]^{2+} + \mathrm{MetalL_2}^- \rightarrow [(\mathrm{Pep} + 2\mathrm{H})\cdots\mathrm{MetalL_2}]^{+*} \rightarrow [\mathrm{Pep} + \mathrm{Metal}]^+ + 2\,\mathrm{HL} \quad (1)$$

where L represents the anion of the salt used to form the negatively charged metal–ligand complex. Note that the reactions are expected to go through a relatively long-lived intermediate that upon breakup loses two molecules of HL. Depending upon the nature of the ligands and the size of the peptide, the lifetime of the intermediate can be sufficiently long to allow for observation of the complex. Also, loss of MetalL from the intermediate can compete with loss of HL. In the case of incorporating two alkali metal ions into a doubly protonated peptide, as is the objective in this work, the following reaction is emphasized:

$$[\mathrm{Pep} + 2\mathrm{H}]^{2+} + \mathrm{Metal_2L_3}^- \rightarrow [(\mathrm{Pep} + 2\mathrm{H})\cdots\mathrm{Metal_2L_3}]^{+*} \rightarrow [\mathrm{Pep} + 2\mathrm{Metal} - \mathrm{H}]^+ + 3\,\mathrm{HL} \quad (2)$$

The extent to which reaction (2) can be used as a practical means for generating di-metalated peptide ions depends upon the ability to form multiply protonated forms of the peptide, the ability to generate useful quantities of $\mathrm{Metal_2L_3}^-$ reagent ions, and the ability to identify ligands that lead to multiple losses of HL, rather than losses of MetalL. For all ion/ion reactions involving multiply protonated polypeptides, there is a possibility for proton transfer at a crossing point [48], which, in this case, constitutes a competing side reaction. It is therefore also desirable to use reagents that lead to minimal proton transfer.

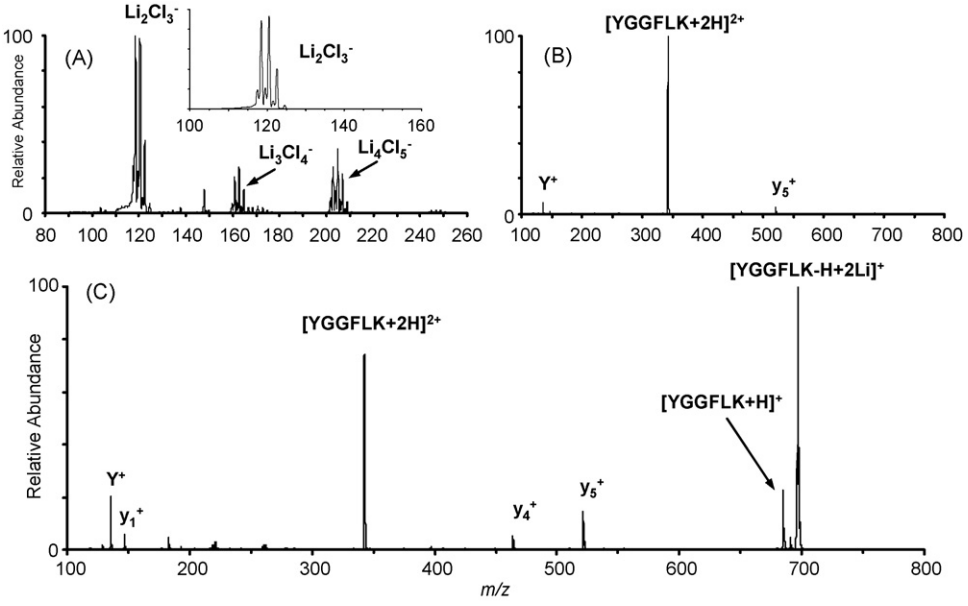

Fig. 1. Ion/ion reaction of $Li_2Cl_3^-$ with $[YGGFLK + 2H]^{2+}$. (A) Negative nano-ESI spectrum of solution of lithium chloride, with inset showing the isolated $Li_2Cl_3^-$ anion. (B) Positive spectrum of isolated doubly protonated peptide $[YGGFLK + 2H]^{2+}$. (C) Ion/ion reaction products of $[YGGFLK + 2H]^{2+}$ and $Li_2Cl_3^-$.

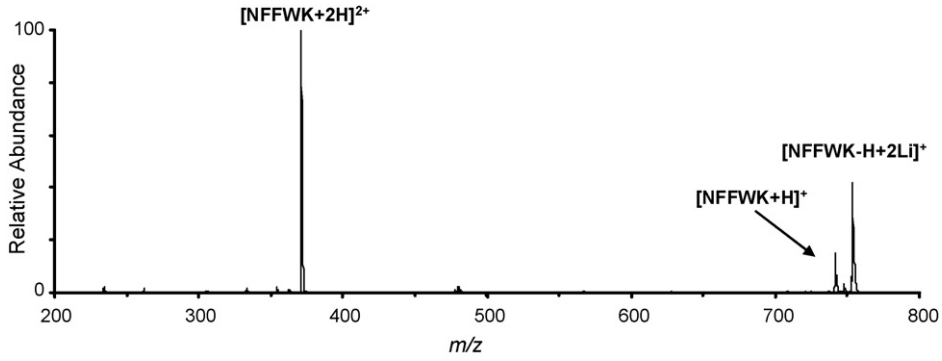

Fig. 2. Ion/ion reaction of $Li_2Cl_3^-$ with $[NFFWK + 2H]^{2+}$.

The data in Fig. 1 summarize a cation switching experiment designed to generate the di-lithiated species $[YGGFLK\text{-}H + 2Li]^+$ from $[YGGFLK + 2H]^{2+}$ using $Li_2Cl_3^-$.

Fig. 1A shows part of the negative nano-ESI mass spectrum of a LiCl solution and illustrates the range of cluster anions that can be formed. In this case, it is straightforward to form $Li_2Cl_3^-$ reactant ions of sufficient abundance for mass selection by Q1. Fig. 1B shows the isolated $(YGGFLK + 2H)^{2+}$ ion prior to the ion/ion reaction. Note the appearance of several fragment ions that are presumably formed upon injection into Q2. A post-ion/ion reaction (positive ion) spectrum is shown in Fig. 1C. It indicates that the major ion/ion reaction pathway is that represented by reaction (2) leading to incorporation of two lithium cations into the peptide. There is also evidence for proton transfer as a relatively minor pathway. The *b*- and *y*-type ions indicated in the spectrum are likely to be unreacted fragment ions already present in the positive ion population prior to ion/ion reaction. Such fragment ions are not observed to be major products in the dissociation of either the $(YGGFLK + H)^+$ or $(YGGFLK\text{-}H + 2Li)^+$ ions (see below). They are, however, major fragments from $(YGGFLK + 2H)^{2+}$ (data not shown).

The results summarized in Fig. 1 appear to be fairly general for relatively small doubly charged peptides. Very similar results for $(NFFWK + 2H)^{2+}$, for example (see Fig. 2) were obtained. In this case, less fragmentation was noted after cation isolation/accumulation than was observed with the YGGFLK system. Likewise, fewer contributions from fragment species are noted in the post-ion/ion reaction data. This is another piece of evidence that suggests little or no fragmentation arising from the ion/ion

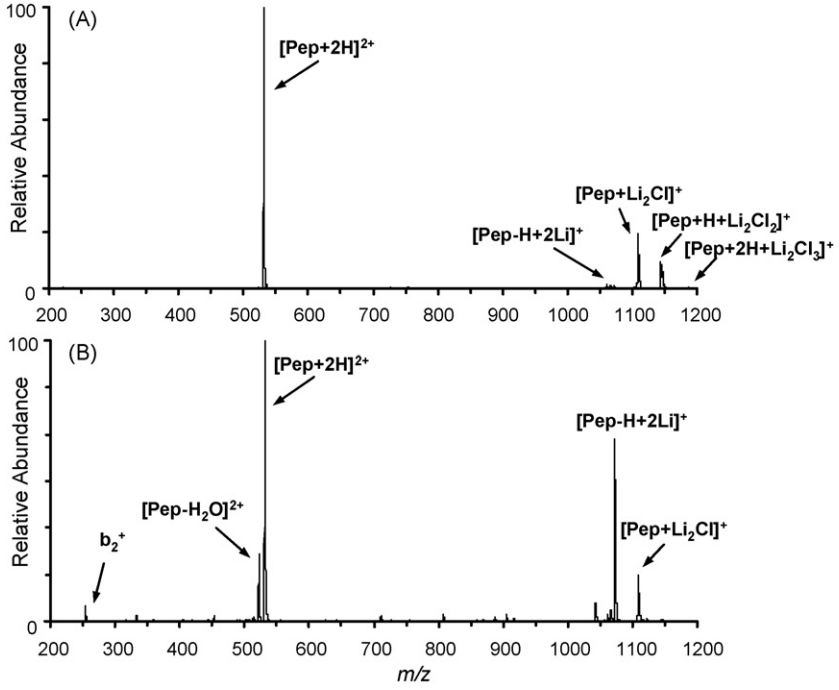

Fig. 3. Comparison of bradykinin post-ion/ion reaction spectra of gentle ion transfer between Q2 and Q3 and beam-type CID between Q2 and Q3. (A) Ion/ion product spectrum (no CID) for bradykinin (RPPGFSPFR). (B) Low energy beam type CID (−12 V) applied after ion/ion reactions to produce greater abundance of di-lithiated bradykinin $[Pep\text{-}H + 2Li]^+$.

reactions. For both the peptides associated with Figs. 1 and 2, proton transfer to yield the singly protonated species was noted and contributed 10–20% to the total ion/ion reaction products and there was no evidence for the intermediate $[(Pep + 2H)\text{-}Li_2Cl_3]^+$ species. Hence, cation switching with $Li_2Cl_3^-$ is a relatively straightforward way to generate di-lithiated ions from these peptides.

For larger doubly protonated polypeptides, evidence for survival of the intermediate ion/ion complex, along with varying degrees of dissociation of the complex, was noted, as illustrated in Fig. 3 with doubly protonated bradykinin, $(RPPGFSPFR + 2H)^{2+}$. Fig. 3A shows the positive ion post-ion/ion reaction data for reaction of doubly protonated bradykinin with $Li_2Cl_3^-$ in Q2 followed by gentle transfer of product ions from Q2 to Q3 and subsequent mass analysis in Q3. A small but detectable signal was observed for the intermediate formed upon combination of the anion and cation (viz., the $(Pep + 2H + Li_2Cl_3)^+$ species). Much more prominent signals were observed for products associated with partial break-up of the ion/ion attachment products. These products arise from a loss of either one or two molecules of HCl. A small signal associated with loss of three HCl molecules (reaction (2)) was also noted. Mild collisional activation of the major ion/ion reaction products observed in Fig. 3A resulted in facile loss of one or two molecules of HCl to yield the di-lithiated peptide ion. Mild collisional activation of the ion/ion reaction products is straightforward to effect in the hybrid linear ion trap/triple quadrupole system used in this work. By increasing the potential differ-

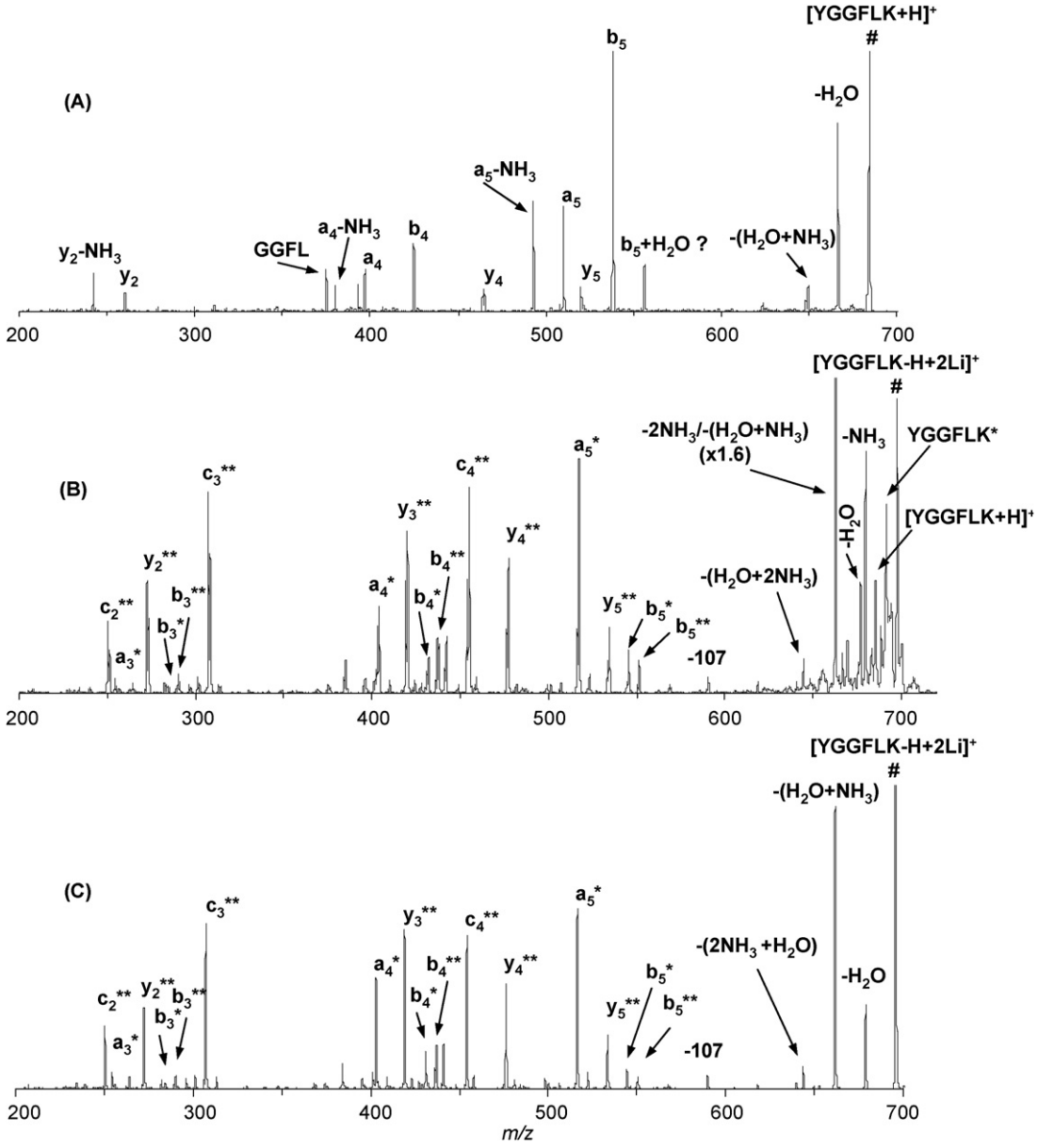

Fig. 4. Activation of YGGFLK from protonated and di-metalated species. The # symbol indicates the precursor ion. (A) CID of $[YGGFLK + H]^+$, (B) CID of $[YGGFLK\text{-}H + 2Li]^+$ formed directly from nano-ESI of solution of metal salt and peptide, (C) CID of $[YGGFLK\text{-}H + 2Li]^+$ formed via ion/ion reaction of isolated $[YGGFLK + 2H]^{2+}$ and $Li_2Cl_3^-$. The 107 Da loss results from loss of *p*-hydroxybenzyl radical.

ence in the dc offset voltages applied to Q2 and Q3 during the ion transfer step, ions can undergo energetic collisions in the pressure gradient between Q2 and Q3 [49]. Fig. 3B shows the spectrum obtained after the ions were transferred from Q2 to Q3 using a dc offset difference of −12 V. In this way, it is straightforward to convert the ion/ion reaction intermediates and their incomplete dissociation products to the di-lithiated peptide ion of interest.

3.2. Activation of di-metalated peptide ions formed via cation switching

A key issue in the use of ion/ion reactions to form di-metalated peptide ions is whether these ions dissociate similarly to ions of the same molecular formula formed directly from electrospray of a solution containing both the metal salts and the peptide. Fig. 4 compares the CID spectra of singly protonated YGGFLK (Fig. 4A) with the di-metalated species of [YGGFLK-H + 2Li]$^+$ formed directly from nano-ESI of a solution of metal salt and peptide (Fig. 4B) and with the fragmentation of [YGGFLK-H + 2Li]$^+$ formed as a result of ion/ion reactions (Fig. 4C). Comparable parent ion signals for the [YGGFLK-H + 2Li]$^+$ species could be generated via the two synthetic routes, although the mass spectrum of the salt/peptide mixture showed significantly more chemical noise. The fragmentation spectra of both of the di-lithiated species are noticeably different from the protonated species. The fragmentation patterns of the two di-lithiated species, however, are very similar to one another. For the protonated species, the dominant fragmentations observed are the losses of ammonia and water, and also the cleavage of the C-terminal lysine to form the b_5 ion. The di-lithiated species formed either from ion/ion reactions or formed directly via nano-ESI show cleavages along the peptide backbone to give c_n^{**}, a_n^* and both b_n^* and b_n^{**} ions. Another observed loss of 107 Da results from homolytic cleavage of the tyrosine residue to produce a *p*-hydroxybenzyl radical. The same loss from YGGFLK was previously reported by Wang in a study of di-lithiated peptides formed by electrospray of peptide solutions which contain metal salts [37].

The similarity in dissociation behavior for the di-metalated peptides formed via different routes does not necessarily indicate that the parent ions share a common structure or mixture of structures because ion trap collisional activation is a slow heating method [50]. However, the results are significant insofar as they indicate that the same structural information can be obtained via either ion formation approach when ion trap collisional activation is used to fragment the ions. A number of other di-lithiated peptide ions have also been generated via ion/ion chemistry and subjected to ion trap collisional activation. In all cases in which ions of the same molecular formula were formed by electrospray of solution containing a mixture of metal salts and peptides, either by ourselves or by others (as reported in the literature), the CID results for the ions are very similar. Examples of ion trap CID data for (Pep-H + 2Li)$^{2+}$ ions formed via the ion/ion reaction route for the peptides VDPVNFK, DRVYIHPF, KGAILKGAILR, and ALILTLVS are provided as supplementary material.

4. Conclusions

The gas-phase cation switching of two lithium cations for three protons has been effected by the ion/ion reaction of $Li_2Cl_3^-$ with doubly protonated peptides to yield the (Pep-H + 2Li)$^+$. For relatively small peptides, the cation switching reaction is the dominant ion/ion reaction channel with minor competition from proton transfer. For larger doubly protonated peptides, the intermediate ion/ion complex and losses of HCl therefrom are also observed. These products can be readily converted to the (Pep-H + 2Li)$^+$ by gentle collisional activation of the products upon transfer from the linear ion trap used for the ion/ion reaction to the linear ion trap used for mass analysis. Ion trap collisional activation of the di-lithiated peptides formed by ion/ion reactions produce product ion spectra that are essentially identical to those produced when the di-lithiated ions are formed via direct electrospray of a solution containing metal salts and the peptide. The ion/ion reaction technique, therefore, provides an alternate means for generating di-lithiated peptide ions. The key advantage of the ion/ion reaction approach is that it obviates mixing of metal salts with peptides of interest in the condensed-phase, which can result in varying degrees of metal ion incorporation and mass spectra characterized by higher degrees of chemical noise than those generated without added metal salts. The ion/ion reaction approach affords a high degree of flexibility in defining precisely the identities of the reactants, relative to solution mixing, and this approach also allows for the independent optimization of ionization conditions for the ion/ion reactants. The ion/ion reaction approach, however, requires that the peptide must be at least a doubly protonated species for the cation switching reaction, although this approach could certainly be used for more highly charged peptide species. This approach also requires an instrument capable of performing ion/ion reactions.

A number of other lines of work can be envisioned as evolving from the study reported here. These include the formation of di-metalated peptides with other cations, the cation switching of more than two metal ions into a polypeptide ion, the formation of peptides with mixtures of cationizing species, or the formation of more highly charged di-metalated ions are a few of the possibilities that have been demonstrated [40,41,43,51]. The flexibility afforded by the use of separate ionization sources for cationic and anionic reactants as well as the capabilities of electrodynamic ion traps to perform a variety of tandem mass spectrometry operations (beam type CID and in-trap CID) make this technique a useful alternative strategy for the formation of di-lithiated peptide ions.

Acknowledgements

This work was supported by the Office of Basic Energy Sciences, Division of Chemical Sciences under Award No. DE-FG02-00ER15105. We acknowledge Dr. Yu Xia for supplying the sample of digested somatostatin-14 (peptide NFFWK), and for many helpful discussions.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijms.2007.02.030.

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