A Study of the Interactions between Group 1 Metals and the C-Terminus of Peptides by Means of High Energy Tandem Mass Spectrometry

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Abstract: Alkali metal−peptide complexes of the type $(M - H + 2Cat)^+$ have been studied by high energy collision-induced dissociation. In addition to structure specific sequence ions, loss of the C-terminal functional group as a radical ion occurs as a prominent fragmentation channel. C-terminal acids generate $(Cat_2CO_2)^{\bullet+}$ and $(Cat_2)^{\bullet+}$ in a fashion similar to the behavior of fatty acids. C-terminal amides generate $(Cat_2NCO)^+$ and $(Cat_2)^{\bullet+}$ which have not been previously discussed. The formation of all of these may be rationalized by a series of consecutive radical reactions. During these fragmentations the sites of metal ion binding are well defined and equally suitable for the attachment of both small and large univalent group 1 metals. Mixed cation systems of the general form $(M - H + Cat_A + Cat_B)^+$ also generate these radical ion species. The binding of two distinct cations to the same peptide allows one to investigate certain competitive loss processes.

Introduction

Mass spectrometry finds many analytical applications in the biological sciences, playing a complementary role alongside the traditional wet techniques of biochemistry. An important contribution has been the combination of fast atom bombardment ionization (FAB) and tandem mass spectrometry in studying the structures of biologically important molecules. The most commonly used techniques are high and low energy collision-induced dissociation experiments (CID) which have proved to be effective approaches in determining the structures of biomolecules including peptides, saccharides, and their derivatives.[1ab]

In the absence of metal salts, FAB tends to generate the protonated form of sample molecule $(M + H)^+$, and many studies have employed this adduct as the precursor ion for MS/MS experiments. In the case of peptides, collisional activation of the $(M + H)^+$ ion produces fragment ions which are related to the amino acid sequence by cleavages along the backbone and side chains.[1a,2]

Peptides and other biological molecules also possess a large affinity for metal cations, and this is shown by the diverse range of metal complexes that have been observed in the gas phase.[3−9] Collisional activation of these metal adducts produces fragment ions which are not commonly observed in the tandem mass spectrum of the corresponding $(M + H)^+$ ion. Therefore, a complementary source of structural information is available from studying these metal complexes.

Much of the work in this area has focused on the collisional activation of the $(M + Cat)^+$ adducts of peptides.[3,10,11] Higher group 1 metal complexes such as $(M - H + 2Cat)^+$ are also prominent in the MS spectrum, although there is little documentation concerning the collision-induced fragmentations of this type of metal−peptide complex.[12,13]

This study investigates the high energy collision-induced dissociation of the $(M - H + 2Cat)^+$ adducts of peptides and concentrates on the fragments produced where both metals are bound to the C-terminal portion of the peptide molecule. Adducts containing a C-terminal carboxylic acid produce ions that have been observed previously from the high energy CID of fatty acid complexes of the type $(M - H + 2Cat)^+$.[14,15] Peptides possessing a C-terminal amide functional group generate an analogous series of ions not previously documented. Other binding sites along the peptide chain are also available to the metal cations, and this results in fragment ions related to the peptide sequence.

The findings are described largely by comparison of the model peptide analogues leucine enkephalin (YGGFL-OH) and leucine enkephalin amide (YGGFL-NH_2), which differ in the C-terminal functional group. In addition, these analogues differ in mass by only 1 Da; thus, any differences in fragmentation are due to the chemical change at the C-terminus rather than to differences in the center of mass collision energy.

Experimental Section

Mass Spectrometry. Collision-induced dissociation experiments were performed using a Kratos Concept IIHH 4-sector instrument of $E_1B_1E_2B_2$ geometry. The samples were ionized using a liquid SIMS

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Table 1. Fragment Ion Abbreviations for Metal Cation−Peptide Complexes

ions from $(M - H + 2Cat)^+$	abbreviation	ions from $(M - H + 2Cat)^+$	abbreviation
$y_n + 2Cat - 2$	y_n	$c_n + 2Cat$	c''_n
$y_n + 2Cat$	y''_n	$a_n + 2Cat - 1$	a_n
$z_n + 2Cat - 1$	z'_n	$d_n + 2Cat - 1$	d_n

cesium ion gun operated at 15 kV and a current of 1 mA, and the ions subsequently formed were accelerated to 8 keV and passed into MS1 (E_1B_1). The desired precursor ion was mass selected at a resolution of approximately 1000 and passed into the collision cell situated in the third-field free region. The collision cell was floated at 4 kV to allow a laboratory impact energy of 4 keV, and an inert collision gas was introduced in order to attenuate the precursor ion to 30% of its initial intensity. For the majority of the results presented helium was employed as the target gas although a comparison of helium and argon is discussed. The remaining intact precursor ions and all product ion fragments were post-accelerated by 4 keV to improve the collection efficiency of low mass ions and reduce the number of ions lost by scattering. MS2 (E_2B_2) was operated in the product ion scan mode. The ions were recorded by a scanning array detector which records 4% of the mass range simultaneously. All data were processed via the MACH 3 data system, and at least 10 summed scans were taken for each experiment.

Sample Preparation. Linear peptides were purchased from Sigma Chemical U.K., cyclic peptides were obtained from Calbiochem Novabiochem U.K. and used without further purification. The alkali metal acetate was applied to the probe tip (1 μL, 0.5M solution in MeOH) followed by the peptide sample (1 μL, 1 nmol μL^{-1} solution in MeOH). The mixed adducts were prepared in the same manner (1 μL, 0.25 M Cat_A + 0.25 M Cat_B solution in MeOH). The matrices used were either 1:1 glycerol:thioglycerol or 5:1 dithiothreitol:dithioerythritol.

Nomenclature classifying fragmentations of MH^+ described by Roepstorff[16] and Biemann[2] can be modified to include fragmentations of metal-cationated peptides.[12] Dissociation of $(M - H + 2Cat)^+$ can occur as shown below:

$$(M - H^+ + 2Cat)^+ = (F_i + 2Cat)^+ + N$$

$$(M - H^+ + 2Cat)^+ = (F_i + Cat)^+ + N$$

where F_i represents the peptide fragment and N the neutral loss. Product ions retaining both cations are abbreviated to aid clarity, as shown in Table 1. Assignments of product ions retaining only one of the metal centers are represented explicitly.

Results and Discussion

Reactions of the Carboxylic Acid Functional Group. High energy CID of the $(M - H + 2Na)^+$ adduct of leucine enkephalin is shown in Figure 1a. This complex generates a range of sequence specific ions including $(c_n + 2Cat)^+$, $(y_n + 2Cat)^+$, $(y_n + Cat - 2H)^+$ and a variety of high mass a_n and d_n species. In addition, two low mass ions of interest are present at *m/z* 90 and 46, which have been rationalized as $(Na_2CO_2)^{\bullet+}$ and $(Na_2)^{\bullet+}$, respectively. This is supported by the corresponding ions formed from $(M - H + 2Cs)^+$ shown in Figure 1b at *m/z* 310 and 266 and from the dicationated adducts of the other alkali metals. Gross and Adams have observed these radical ions from the CID of fatty acid complexes of the type $(M - H + 2Cat)^+$, [14,15] where the carboxylic acid group is the major binding site. In the case of peptides there are many more polar functional groups present, and hence, a whole range of sites is possible for metal attachment. Even with a large number of other binding sites present the C-terminal carboxylic acid

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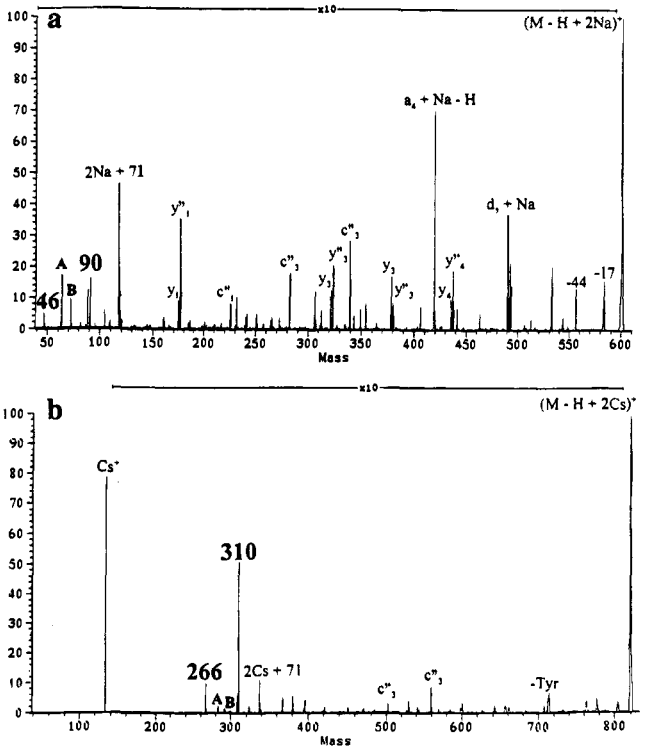
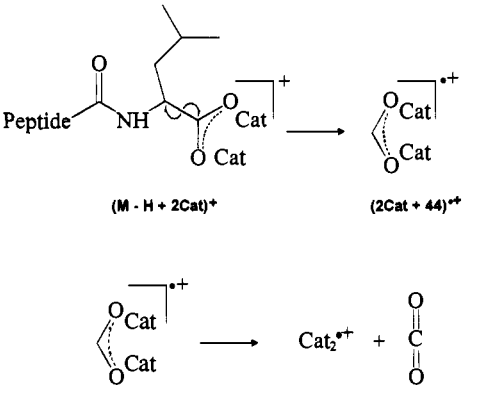


Figure 1. (a) High energy CID spectrum of disodiated leucine enkephalin, $(M - H + 2Na)^+$, A = $(Na_2OH)^+$, B = $(Na_2CN)^+$. (b) High energy CID spectrum of dicesiated leucine enkephalin, $(M - H + 2Cs)^+$, A = $(Cs_2OH)^+$, B = $(Cat_2CN)^+$.

Scheme 1. Proposed Mechanism for the Generation of $(Cat_2CO_2)^{\bullet+}$ from the C-Terminus



remains an important site for metal−peptide interactions. The presence of the y-type sequence ions and $(a_4 - H + Cat)^+$ where a metal ion is lost along with the C-terminus also suggests that the C-terminal portion of the molecule is the major binding site. The c-ions are the only major series which indicates another site of interaction between the metal and the peptide.

Energetic and Mechanistic Considerations. A mechanism for the formation of these adducts is given in Scheme 1. Both attached cations must be closely associated with the deprotonated C-terminus. High energy collisional activation results in cleavage of the carbon−carbon bond adjacent to the C-terminus, and from this reaction the fragment $(Cat_2CO_2)^{\bullet+}$ is generated with a corresponding loss of the rest of the peptide as a large neutral radical. Some stabilization of the $(Cat_2CO_2)^{\bullet+}$ adduct will be gained by delocalization of the unpaired electron and charge over the carboxylate group. Another limiting form for the structure of this product would be fully covalent such as $(Cat\text{-}CO_2Cat)^{\bullet+}$, corresponding to a metallic version of formic acid. Further dissociation of the $(Cat_2CO_2)^{\bullet+}$ adduct occurs,

Table 2. Ratio of $(Cs_2CO_2)^{\bullet+}/(Cs_2)^{\bullet+}$ as a Function of Collision Cell Pressure for Leucine Enkephalin at a Laboratory Collision Energy of 4 keV

$(Cs_2Co_2)/(Cs_2)$	pressure/Torr	% transmission
7.2	4×10^{-7}	70
6.9	6×10^{-7}	50
5.9	9×10^{-7}	30
4.4	1×10^{-6}	15

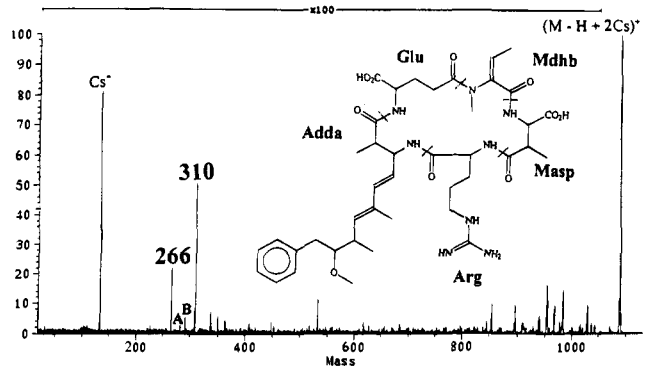


Figure 2. High energy CID spectrum of dicesiated nodularin, $(M - H + 2Cs)^+$, A = $(Cs_2OH)^+$, B = $(Cs_2CN)^+$, Adda = 3-amino-9-methoxy-2,6,8-trimethyl-4,6-decadienoic acid, Mdhb = *N*-methyldehydroamine-2-butyric acid.

which results in the formation of carbon dioxide as a neutral and the metal radical dimer ion $(Cat_2)^{\bullet+}$.

Low energy collisional activation of peptide complexes of the type $(M - H + 2Li)^+$ generates many of the sequence specific ions[12,13] observed at the higher energies employed in this study and suggests that sequence ions occur by low energy pathways. The $(Li_2CO_2)^{\bullet+}$ ion at *m/z* 58 was not observed, and this implies that the formation of this radical complex is a higher energy process.

Variation of the ratio $(Cs_2CO_2)^{\bullet+}/(Cs_2)^{\bullet+}$ was measured as a function of collision gas pressure at a constant laboratory collision energy of 4 keV. The gas pressure was adjusted to allow transmissions of the precursor ion beam of 70, 50, 30 and 15% as shown in Table 2. Dissociation of the $(Cat_2CO_2)^{\bullet+}$ ion to $(Cat_2)^{\bullet+}$ is more favored at higher collision gas pressures shown by a decrease in the ratio of $(Cs_2CO_2)^{\bullet+}/(Cs_2)^{\bullet+}$ from a transmission of 70% to 15%, and this suggests that the reaction has a high activation energy.

Metal complexes of cyclic peptides may also generate $(Cat_2CO_2)^{\bullet+}$ and $(Cat_2)^{\bullet+}$ although it is necessary to have a pendant carboxylic acid present. An example of this is shown for the $(M - H + 2Cs)^+$ complex of nodularin in Figure 2 where the γ-Glu and β-MeAsp residues provide the $-COOH$ side chains. The $(M - 2H + 3Cat)^+$ adducts of this peptide also generate the $(Cat_2CO_2)^{\bullet+}$ and $(Cat_2)^{\bullet+}$ product ions upon collisional activation. Other aspects of metal cation attachment to cyclic systems will be published at a later date.

Collisional Activation of Complexes of the Type $(M - H + Cat_A + Cat_B)^+$. MS/MS studies of dicationized adducts of the form $(M - H + Cat_A + Cat_B)^+$ were also performed where Cat_A and Cat_B are different alkali metal cations. These were prepared by adding equimolar quantities of the metal acetate salts to the probe tip which gives the optimum yield of the mixed adduct with respect to the other possible complexes, i.e., $(M + Cat_A)^+$, $(M + Cat_B)^+$, $(M - H + 2Cat_A)^+$, and $(M - H + 2Cat_B)^+$. Figure 3a shows the CID spectrum of the adduct $(M - H + K + Cs)^+$ which dissociates to give $(KCsCO_2)^{\bullet+}$ and $(KCs)^{\bullet+}$ as the major dissociation products.

Mixed adducts allow the two binding cations to be "labelled" separately, and competitive effects between the different cations

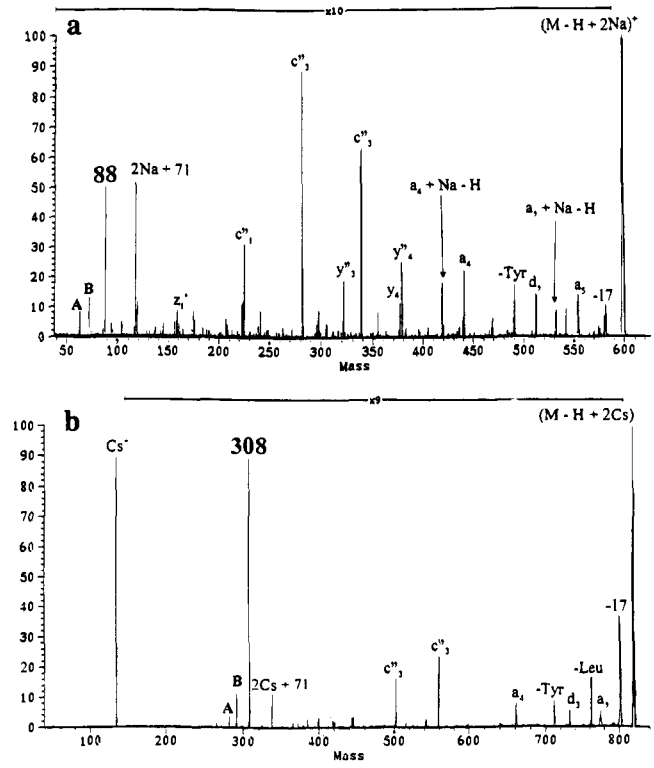


Figure 3. (a) High energy CID spectrum of the $(M - H + K + Cs)^+$ precursor ion of leucine enkephalin, A = $(KCsOH)^+$, B = $(KCsCN)^+$. (b) High energy CID spectrum of the $(M - H + Li + Na)^+$ precursor ion of leucine enkephalin. Upper mass range shown.

can be studied. This is shown in Figure 3a where loss of the bare Cs^+ cation is greater than the loss of K^+ from the same complex, supporting the view that the binding interaction between the peptide and the alkali metal weakens on descent of the group 1 series. Other examples of competitive fragmentations are shown in Figure 3b from the $(M - H + Li + Na)^+$ precursor ion. The $(d_5 + Cat)^+$ product ion formed from $(M - H + 2Cat)^+$ occurs by loss of the C-terminal portion of the peptide, the leucine side chain, and one of the metal cations. In a mixed adduct system two ions can form, $(d_5 + Li)^+$ and $(d_5 + Na)^+$, of which the lithiated fragment ion is more abundant. The same trend is observed for the $(a_5 + Li - H)^+$ and $(a_5 + Na - H)^+$ products. This appears to show that the larger cation is lost in preference, and two reasons could account for this observation. The larger cation may bind preferentially to the C-terminus, which is lost during the formation of the a_5- and d_5-type ions. Alternatively, the greater polarizing effect of the smaller sized cation may favor other reaction channels. The $(M - H + Li + Na)^+$ complex produces another doublet corresponding to $(a_4 + Li - H)^+$ and $(a_4 + Na - H)^+$, the more abundant being the sodiated fragment. This is in contrast to the previous example and indicates that cation size and binding regime are very important factors in determining the ion abundance.

The mixed series $(M - H + Li + Cat_B)^+$ undergoes collisional activation to yield the carboxylate species $(LiCat_B-CO_2)^{\bullet+}$, but no $(LiCat_B)^{\bullet+}$ dimer is observed which may relate to the size/orbital mismatch between lithium and the other alkali metals. Gross and Adams have also noted that only the $(M - H + 2Li)^+$ complexes of the smaller fatty acids generate the $(Li_2)^{\bullet+}$ ion upon high energy collisional activation.[14] This suggests that the binding interaction of the lithium cation to the radical carboxylate species is strong.

Reactions of the C-terminal Amide Functional Group. MS/MS of leucine enkephalin amide is shown in Figure 4a, b

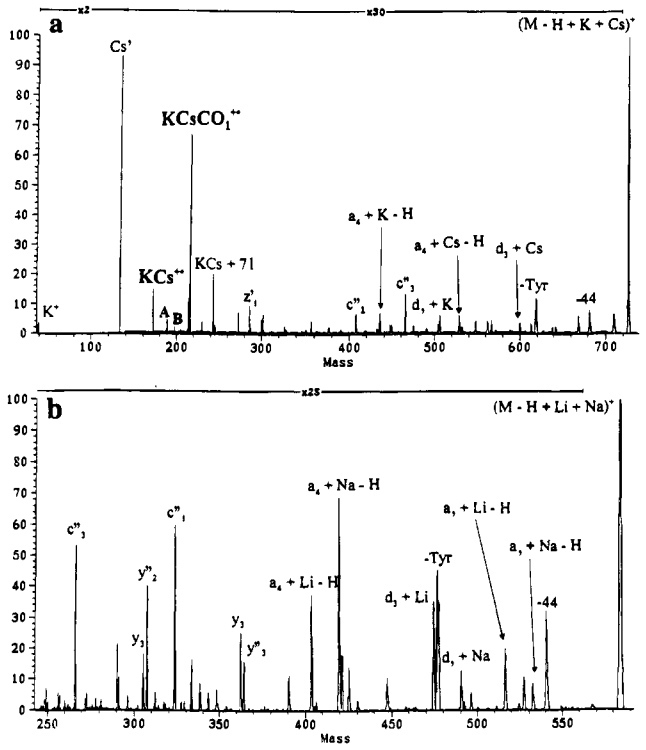
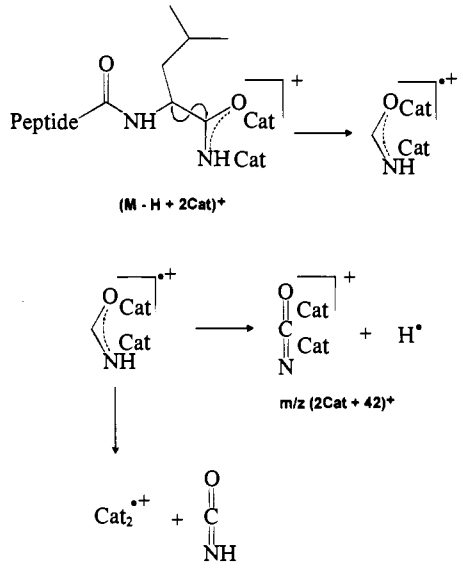


Figure 4. (a) High energy CID spectrum of the disodium complex of leucine enkephalin amide, $(M - H + 2Na)^+$, A = $(Na_2OH)^+$, B = $(Na_2CN)^+$. (b) High energy CID spectrum of the dicesium complex of leucine enkephalin amide, $(M - H + 2Cs)^+$, A = $(Cs_2OH)^+$, B = $(Cs_2CN)^+$.

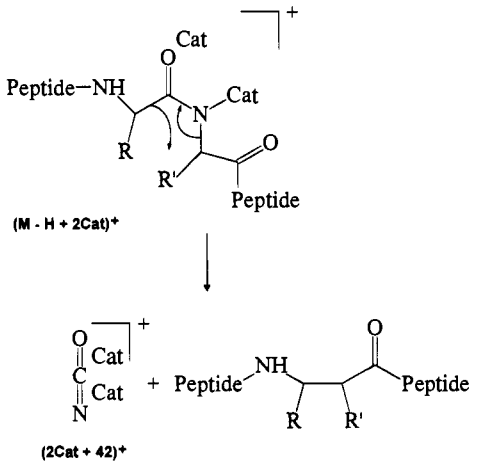
Scheme 2. Proposed Mechanism for the Generation of $(Cat_2NCO)^+$ from the C-Terminus



for the complexes $(M - H + 2Na)^+$ and $(M - H + 2Cs)^+$, respectively. Both precursor ions yield one fragment ion of the type described previously, at *m/z* 88 from the $(M - H + 2Na)^+$ adduct and 308 from the $(M - H + 2Cs)^+$ adduct, and are assigned to the general formula $(2Cat + 42)^+$. Two possible mechanisms for the formation of this entity are given in Schemes 2 and 3.

In Scheme 2 both cations are closely associated with the C-terminus via deprotonation of the amide function. Upon collisional activation the carbon−carbon bond adjacent to the metal binding site undergoes homolytic fission to generate

Scheme 3. Proposed Mechanism for the Generation of $(Cat_2NCO)^+$ from a Backbone Amide Group



$(HNCOCat_2)^{\bullet+}$. The structure of this radical ion resembles the $(z_1 + Cat^{II} - H)^{\bullet+}$ species formed from group 2 metal-peptide complexes.[4]

Dissociation of the dicesiated complex by this pathway would result in the formation of the $(HNCOCs_2)^{\bullet+}$ species at *m/z* 309, 1 Da higher in mass than the observed product. Loss of hydrogen from the radical complex as H• would provide a facile, low energy pathway to account for this difference. Furthermore, the product ion formed would be the thermodynamically stable isocyanate group to which the two metal cations are bound.

With helium as the collision gas, the dimer $(Cat_2)^{\bullet+}$ is not prominent when the C-terminal functional group is an amide. Scheme 2 shows that loss of H• to form $(Cat_2NCO)^+$ and generation of $(Cat_2)^{\bullet+}$ by the elimination of HNCO occurs from the same fragment ion and so are in direct competition. Loss of a hydrogen radical from the $(Cat_2HNCO)^{\bullet+}$ complex appears to be the more favored process. In addition, the production of the metal radical dimer is accompanied by a neutral loss of HNCO, the conjugate acid of the isocyanate species. The instability of isocyanic acid may inhibit the reaction channel which forms $(Cat_2)^{\bullet+}$.

These results also suggest that the formation of $(Cat_2)^{\bullet+}$ does indeed occur from the C-terminal functional group. If $(Cat_2)^{\bullet+}$ were formed at a region of the molecule remote from the C-terminus, both the acid and amide analogues of leucine enkephalin would give rise to this radical product at the same center of mass collision energy.

Use of argon as the collision gas in place of helium increases the centre of mass collision energy while retaining the same laboratory collision energy of 4 keV. The $(M - H + 2Cat)^+$ adducts of the leucine enkephalin amide generate both $(Cat_2NCO)^+$ and $(Cat_2)^{\bullet+}$ on activation by the heavier target gas, and this supports the conclusion that the formation of the metal dimer radical is a high energy process.

Other amides may also undergo this reaction, such as the $(M - H + 2Cat)^+$ adduct of $H_2NOC(CH_2)_8CONH_2$ which generates both $(Cat_2NCO)^+$ and $(Cat_2)^{\bullet+}$ with helium as the collision gas. A fragment ion corresponding in mass to the formula $(Cat_2HNCO)^{\bullet+}$ is also observed in low abundance.

A second mechanism for the generation of the $(2Cat + 42)^+$ ion is proposed in Scheme 3 from another isomer of $(M - H + 2Cat)^+$, where the two metal cations are associated with a deprotonated amide along the peptide backbone. Collisional activation of this isomer of the complex could lead to cleavage of the two bonds about the amide group, i.e., the CH(R)−CO and N-CH(R′) bonds, and such a reaction would yield the $(Cat_2NCO)^+$ ion directly.

Complexes of C-terminal acids yield a small amount of the $(Cat_2NCO)^+$ adduct 2 Da lower in mass than $(Cat_2CO_2)^{\bullet+}$ which would be generated by this second mechanism, while complexes of C-terminal amides could dissociate by both of the pathways described.

The product ions generated by the amide functional group $(Cat_2HNCO)^{\bullet+}$, $(Cat_2NCO)^+$ and $(Cat_2)^{\bullet+}$, have not been reported previously, although they are related to the odd electron ions $(Cat_2CO_2)^{\bullet+}$ and $(Cat_2)^{\bullet+}$ formed from the $-COOH$ group as first proposed by Gross.[14,15]

Other Fragment Ions/Binding Sites. Both N-terminal and C-terminal sequence specific ions are produced by these dicationized complexes, the most common series being $(y_n + 2Cat)^+$, $(y_n + 2Cat - 2H)^+$, $(c_n + 2Cat)^+$ and various a_n- and d_n-type ions. These fragments are all generated from the same precursor ion $(M - H + 2Cat)^+$, yet it would be difficult to assign a single structure from which both N- and C-terminal fragments could be formed. It is likely that a range of binding positions are open to the cations, and therefore, a population of precursor ion isomers are present. Adams has shown that for group 2 metal complexes the relative importance of a sequence specific ion is a function of the amino acids present, the size of the binding cation, and the C-terminal functional group, all of which will affect the proportions of individual isomers.[4]

Other low mass species observed include $(Cat_2OH)^+$ and $(Cat_2CN)^+$ denoted as A and B on the spectra. Bouchonnet has noted similar adducts derived from the simple system $(GlyCat)^+$ from plasma desorption ionization.[17] Both analogues of leucine enkephalin generate these species which suggests that they originate from dissociations along the backbone of the peptide. Another fragment ion occurs for both the acid and amide analogue at a mass of (2Cat + 71), which also suggests that this species occurs from along the backbone of the peptide. The $(2Cat + 71)^+$ ion does not appear to be associated with a specific amino acid residue and is also observed in the tandem mass spectrum of nodularin and other peptides such as Pro-Pro-Gly-Phe-Ser-Pro and Tyr-Phe-Met-Arg-Phe-NH_2. The origin of this ion is not fully understood.

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Conclusions

The large number of polar functional groups along the peptide provides a number of sites to which metal cations may bind. A population of cationized isomers exists which will fragment according to the structure of the complex and therefore generate a variety of fragment ions. Results suggest that the formation of $Cat_2CO_2^{\bullet+}$ from the C-terminal acid and Cat_2NCO^+ from the C-terminal amide occur from complexes where the two cations are located at the C-terminus. The site of the cation–peptide interaction is well defined for the generation of these odd electron ions, in contrast to many of the pathways proposed for the formation of sequence specific ions. In addition, the presence of carboxylic acid and amide functions can be distinguished by the mass difference of their C-terminal product ions, i.e., $Cat_2CO_2^{\bullet+}$, *m/z* $(2Cat + 44)^+$, and Cat_2NCO^+, *m/z* $(2Cat + 42)^+$.

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