Generation of Peptide Radical Dications via Low-Energy Collision-Induced Dissociation of $[\text{Cu}^{\text{II}}(\text{terpy})(\text{M} + \text{H})]^{3+}$

Ivan K. Chu and Corey N. W. Lam

Department of Chemistry, The University of Hong Kong, Hong Kong, China

The first example of the formation of hydrogen-deficient radical cations of the type $[\text{M} + \text{H}]^{2+}$ is demonstrated to occur through a one-electron-transfer mechanism upon low-energy collision-induced dissociation (CID) of gas-phase triply charged $[\text{Cu}^{\text{II}}(\text{terpy})(\text{M} + \text{H})]^{3+}$ complex ions (where M is an angiotensin III or enkephalin derivative; terpy = 2,2':6',2''-terpyridine). The collision-induced dissociation of doubly charged $[\text{M} + \text{H}]^{2+}$ radical cations generates similar product ions to those prepared through hot electron capture dissociation (HECD). Isomeric isoleucine and leucine residues were distinguished by observing the mass differences between $[z_n + \text{H}]^+$ and w_n^+ ions (having the same residue number, n) of the Xle residues. The product ion spectrum of $[z_n + \text{H}]^+$ reveals that the w_n^+ ions are formed possibly from consecutive fragmentations of $[z_n + \text{H}]^+$ ions. Although only the first few $[\text{M} + \text{H}]^{2+}$ species have been observed using this approach, these hydrogen-deficient radical cations produce fragment ions that have more structure-informative patterns and are very different from those formed during the low-energy tandem mass spectrometry of protonated peptides. (J Am Soc Mass Spectrom 2005, 16, 1795–1804) © 2005 American Society for Mass Spectrometry

The dissociation pathways of protonated species have been studied extensively and general rules governing their fragmentation in the gas phase are fairly well characterized [1–3]. The capture of electrons by protonated peptides, which results in electron capture dissociation (ECD) [4–6] and hot electron capture dissociation (HECD) [7], is a viable methods for converting a multiply protonated peptides into a protonated peptide radical cations [4–6, 8, 9]. Previously, this approach could be employed only by using a Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR MS), but recently, a similar technique, which relies on electron-transfer, has been demonstrated on commercial ion trap instruments [10, 11] and has attracted considerable interest. The formation of protonated peptide radical cations through the latter approach is very attractive for the identification of peptides and proteins because it provides very different and more structure-informative dissociation patterns [5, 12–14].

The dissociation of peptide radical cations of the form $[\text{M} + n\text{H}]^{(n-1)+}$ and $[\text{M} + n\text{H}]^{(n+1)+}$ is significantly different from the dissociation of the corresponding even-electron protonated peptide ions $[\text{M} + n\text{H}]^{n+}$. In contrast to protonated peptides, which fragment

predominantly by the cleavage of a peptide bond to form y and b ions, the dissociation of protonated peptide radical cations proceeds through cleavage of an $\text{N}-\text{C}_\alpha$ bond to form predominantly c and z product ions [15, 16]; this difference may allow novel mass spectrometric approaches to be developed for the identification and characterization of biological molecules [15, 17, 18]. Fragmentation of those odd-electron peptide radical cations provides an analytical means for de novo sequencing, especially for locating post-translational modifications [18–23]. It also allows the differentiation between isomeric leucine and isoleucine residues in oligopeptides [24, 25]; this phenomenon is important because the identification of post-translationally modified peptide residues and the differentiation of isobaric leucine and isoleucine (Xle) residues are analytical challenges for tandem mass spectrometry [15, 16]. For example, Zubarev et al. recently demonstrated the unambiguous identification and differentiation of Xle residues through HECD [24, 25].

Despite some recent advances in the preparation of protonated peptide radical cations, their reactivity and fragmentation mechanisms are not well understood. The nonergodic dissociation mechanism (i.e., without intramolecular vibrational redistribution before unimolecular fragmentation) postulated for relatively large biomolecules remains under investigation and debate [15, 26–29], but the findings are of fundamental importance to biological tandem mass spectrometry [30]. Comprehensive studies of these systems have been hindered by difficulties in

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Address reprint requests to Dr. I. K. Chu, Department of Chemistry, The University of Hong Kong, Pokfulam Road, Hong Kong, China. E-mail: ivankchu@hku.hk

Figure 1. Full scan spectrum of $[\text{Cu}^{\text{II}}(\text{terpy})(\text{M} + \text{H})]^{3+}$, where $\text{M} = \text{RVYVHPI}$. The zoom scan spectra of $[\text{Cu}^{\text{II}}(\text{terpy})(\text{M} + \text{H})]^{3+}$ and $[\text{Cu}^{\text{II}}(\text{terpy})\text{M}]^{2+}$ are presented in the inset.

generating gas-phase hydrogen-deficient peptide radical cations and the need for special types of instruments. To the best of our knowledge, only a few reports have been published so far that describe studies of the fragmentation of these odd-electron peptide radical cations, which have been generated mainly by using two different approaches: high-energy collisional excitation with a target gas through electron-transfer within an accelerator mass spectrometer [31] and electron capture dissociation in an FTICR [32]. More recently, McLafferty et al. discovered ECD-type fragment ions upon electrospraying a solution of cytochrome *c* in the absence of electron injection. The protein radical fragments and the reduced iron(II) were proposed to be the products of an intermolecular electron-

transfer process between the iron center and the protein [33]. An attempt to dissociate $[\text{Cu}^{\text{II}}(\text{amine})\text{M}]^{2+}$ complexes was reported to generate peptide radical cations and the reduced copper(I) amine through one-electron-transfer under low-energy collision [34–40]. It is expected that the dissociation of hydrogen-deficient cations of the type $[\text{M} + n\text{H}]^{(n+1)+}$ would be similar to that of the corresponding peptide radical cations M^+ . In terms of their classification, hydrogen-deficient cations $[\text{M} + n\text{H}]^{(n+1)+}$ are not classical molecular radical cations: the former contain extra protons. In this study, we aimed to mimic the formation of radical species of the type $[\text{M} + n\text{H}]^{(n+1)+}$ under low-energy CID of a copper(II)-amine-peptide complex. We attempted to generate

Figure 2. MS^2 spectrum of $[\text{Cu}^{\text{II}}(\text{terpy})(\text{M} + \text{H})]^{3+}$ with open resolution, where $\text{M} = \text{RVYVHPI}$. The amplitude of the resonance excitation RF voltage was 0.70 eV; helium was used as the collision gas.

hydrogen-deficient peptide radical dications $[M + H]^{2+}$ using the aforementioned electron-transfer approach on a commercially available tandem mass spectrometer.

Studies of $[M + H]^{2+}$ species may help us to obtain a better understanding of the gas-phase fragmentation of hydrogen-deficient peptide radical cations. We have conducted the first study to identify the low-energy fragment ions of a series of angiotensin III derivatives, which differ only by the nature of their N- or C-terminal amino acid, and then examined the dissociation of the hydrogen-deficient radical cations of the type $[M + H]^{2+}$. Hopefully, we will be able to extend this emerging field to the broader research community by using conventional tandem mass spectrometers, such as triple-quadrupole, ion trap, or hybrid-quadrupole time-of-flight instruments.

Experimental

Mass Spectrometry

All mass spectrometry experiments were conducted using a quadrupole ion trap mass spectrometer (Finnigan LCQ, ThermoFinnigan, San Jose, CA). Samples typically comprised 600 mM Cu(II) complex and 50 mM peptide in a water/methanol (50:50) solution. These samples were infused continuously at a typical rate of 5 μ L/min into the pneumatically assisted electrospray probe using air as the nebulizer gas. CID spectra of $[Cu(II)(amine)M+H]^{3+}$ complexes were acquired using helium as the collision gas. The injection and activation times for CID in the ion trap were 200 and 30 ms, respectively, and the amplitude of the excitation was optimized for each experiment.

Materials

All chemicals were obtained commercially (Aldrich and Sigma, St. Louis, MO; Bachem, King of Prussia, PA). Angiotensin and leucine enkephalin derivatives were synthesized according to literature procedures [41]. The copper(II)-terpy complexes (terpy = 2,2':6',2''-terpyridine) were synthesized according to the experimental procedure described by Henke et al. [42].

Results and Discussion

Figure 1 displays a typical electrospray mass spectrum obtained by mixing the heptapeptide angiotensin III-Ile and $[Cu^{II}(\text{terpy})]^{2+}$ complex in water/methanol [where M refers to angiotensin III-Ile (RVYVHPI) and L refers to terpy]. The predominant peaks are those for the protonated monomer and dimer of M (m/z 442.3 and m/z 883.5, respectively), the doubly charged complex $[Cu^{II}(\text{terpy})M]^{2+}$ (m/z 589.3), and the triply charged complexes $[Cu^{II}(L)(M + H)]^{3+}$ (m/z 393.2). The assignment of the charge states of these ions was readily accomplished through analysis of the spacing of their isotopic distributions; for example, see the inset of

Table 1. Sequences and relative abundances of $[M + H]^{2+}$ and M^+ ions formed in this study

Sequence (M)	$[Cu(\text{terpy})(M + H)]^{3+} \rightarrow [Cu(\text{terpy})]^{+} + [M + H]^{2+}$ m/z (relative abundance of $[M + H]^{2+}$, %)
RVYVHPI	441.6 (93)
RVYVHPL	441.6 (97)
RAYVHPI	427.5 (100)
RAYVHPL	427.6 (100)
RVYVHPF	458.6 (100)
RVYIHPF	465.7 (56)
KVYVHPI	427.5 (63)
KAYVHPL	413.5 (80)
RLYVHPI	448.6 (100)
RLYVHPL	448.6 (100)
RIYVHPL	448.6 (100)
RIYVHPI	448.5 (80)
RLYIHPI	455.6 (100)
RIYLHPI	455.6 (90)
RPYVHPI	440.5 (100)
YGGFLK	342.1 (7)
YGGFIK	342.1 (14)
YAGFIK	349.1 (4)
AVYVHPI	*
QVYVHPI	*
YGGFLR	*
YAGFLR	*

*No $[M + H]^{2+}$ species were observed; instead, product ions from competitive reaction pathways, such as proton abstraction and peptide fragmentation, predominated.

Figure 1 for the spacings observed for the $[Cu^{II}(L)(M + H)]^{3+}$ and $[Cu^{II}(L)M]^{2+}$ ions. Figure 2 presents a typical low-energy CID spectrum of $[Cu^{II}(L)(M + H)]^{3+}$; electron-transfer from the copper complex to the protonated polypeptide during collisional dissociation results in the generation of the most abundant one-electron-oxidized product ion $[M + H]^{2+}$ at m/z 441.6 and the corresponding reduced product ions of the $[Cu^I(L)]^{+}$ complex at m/z 296.3. The other predominant fragment ion at m/z 388.7 arose from consecutive fragmentations from the $[M + H]^{2+}$ ion (see discussion below). Eighteen of the 22 $[M + H]^{2+}$ ions were generated quite efficiently from the two series of medium-sized peptides. Table 1 displays the effect of various angiotensin and leucine enkephalin derivatives (M) have toward the production of $[M + H]^{2+}$ species in the gas-phase. It is apparent that the formation of $[M + H]^{2+}$ ions from the $[Cu^{II}(\text{terpy})(M + H)]^{3+}$ complexes ions is more prominent for relatively long oligopeptides that present a basic amino acid residue at either the C- or N-terminus; in other cases, competitive reaction pathways—other than the formation of $[M + H]^{2+}$ ions through one-electron-transfer—predominate. Our approach is similar to that employed in the formation of M^+ ions using copper(II) complexes [7, 34–40]. It has been demonstrated that a wide variety of peptide radical cations (M^+) can be produced this way by altering the structure of the auxiliary ligand on the copper atom. Electron-transfer from the peptide to the $[Cu^{II}(\text{amine})]^{2+}$ complex may not always be more fa-

Figure 3. MS³ spectra with unimass resolution, of (a) $[M+H]^{2+}$, (b) M^+ , (c) $[M+H]^+$, and (d) $[M+2H]^{2+}$ where $M = RYVHPHPI$. The amplitudes of the resonance excitation RF voltage were 0.82, 1.84, 0.88 and 2.08 eV, respectively; helium was used as the collision gas. The MS³ spectrum of $[M+H]^{2+}$ after isolation—without turning on the auxiliary radiofrequency generator for dipolar excitation—is presented in the inset to Figure 3a. The MS² spectrum of $[Cu^{II}(\text{terpy})M]^{2+}$ is presented in the inset to Figure 3b.

vorable than the competitive reactions. The ligand should be one that binds strongly to the copper center—otherwise, it will be displaced through a proton abstraction pathway: e.g., $[Cu^{II}(L)M]^{2+} \rightarrow [Cu^{II}(M-H)]^+ + [L+H]^+$ when $L = \text{diethylenetriamine (dien)}$. The

terpy ligand seems to be a good candidate for initial investigations [35, 37–40].

Figure 3a presents a low-energy CID spectrum of the $[M+H]^{2+}$ ion. The intact parent ions of $[M+H]^{2+}$ after isolation are presented in the inset; they were

Figure 3. Continued

obtained before turning on the auxiliary radiofrequency generator for dipolar excitation and they remained stable without fragmentation after trapping for 100 ms. Under resonance excitation, the most abundant fragment ions of $[M + H]^{2+}$ were those arising from (1) facile charged-induced side-chain cleavages of the tyrosine (loss of *p*-quinomethide, $CH_2 = C_6H_4 = O$) [35, 37], valine, isoleucine, and arginine residues, (2) neutral loss of water and carbon dioxide, and (3) fragment ions of $[b_n - H]^+$, a_n^+ , y_n^+ , $[z_n + H]^+$, and w_n^+ ions. The minor fragment ions from the $[M + H]^{2+}$ dications of angiotensin III-Ile (RVYVHPI) also gave (1) a series of low-abundance complementary pairs product ions. (For example, peptide fragmentation of $[M + H]^{2+}$ yields y_2^+ at m/z 229.1 and its complementary fragment $[b_7 - H]^+$ at m/z 654.1: $[M + H]^{2+}$ at m/z 441.6 = 883.2 Da = $y_2^+ + [b_7 - H]^+ = 229.1 + 654.1$ Da; y_5^+ at m/z 628.1/ $[b_2 - H]^+$ at m/z 255.1) through the charge separation on the backbone amide bond cleavage of $[M + H]^{2+}$ and (2) C-terminal fragment $[z_n + H]^+$ ions that resulted from N- C_α bond cleavage, except for the N-terminal proline residue ($[z_2 + H]^+$). The presence of a proline residue retards the cleavage of the N-terminal

N- C_α bond of the proline residue during ECD, HECD, and high-energy collisional excitation [4, 7, 17]. Indeed, the presence of the proline residues is consistent with the absence of $[z_2 + H]^+$ ions from dissociation of the radical dicationic peptide $[M + H]^{2+}$. In contrast, we observed no N-terminal complementary fragment c_n^+ ions from the dissociation of this doubly charged $[M + H]^{2+}$ ion. Figure 3b–d provide a partial comparison of the product ion spectra of three different types of ions obtained from the same heptapeptide RVYVHPI: $[M + 2H]^{2+}$, $[M + H]^+$ and M^+ . It is not surprising that low-energy CID of the even-electron protonated peptide ions of $[M + 2H]^{2+}$ and $[M + H]^+$ leads to the formation of conventional fragment ions of a_n^+ , b_n^+ , and y_n^+ . In contrast, dissociation of the odd-electron peptide radical cations M^+ leads to the formation of fragment ions a_n^+ , y_n^+ , $[z_n + H]^+$, and w_n^+ ; This fragmentation is similar to that of the dissociation of odd-electron peptide radical dications $[M + H]^{2+}$, but is significantly different from those of the corresponding $[M + 2H]^{2+}$ and $[M + H]^+$. We are just beginning to understand and identify the product ions formed from $[M + H]^{2+}$ species under low-energy CID.

Figure 4. MS³ spectrum of $[M + H]^{2+}$ with unimass resolution, where $M = \text{RAYVHPI}$. The amplitude of the resonance excitation RF voltage was 0.79 eV; helium was used as the collision gas.

By replacing valine with alanine at the second amino acid residue of $[M + H]^{2+}$ dications of angiotensin III-Ile (RVYVHPI), we reconfirmed the assignments of the N-terminal fragment ions (i.e., a_n^+ and b_n^+) by observing a mass shift of m/z 28 from the residue mass difference between valine and alanine. In contrast, the fragment ions from the unsubstituted portion should present the same mass-to-charge ratio. The fragmentation pattern in the product ion spectrum of RAYVHPI radical dications displayed in Figure 4 is virtually identical to that in Figure 3a, as expected from the similar structures of these two heptapeptides, which differ only in an additional methyl group at the β

carbon atom of the second amino acid residue. We have also made a similar confirmation of the C-terminal fragment ions (y_n^+ and $[z_n + H]^+$) from the product ion spectrum of the angiotensin III-Phe (RVYVHPF) derivative by deliberately replacing the C-terminal amino acid residue from isoleucine to phenylalanine (Figure 5).

Another striking feature of HECD is that it is a promising method for differentiating between isomeric leucine and isoleucine residues in polypeptides through an analysis of the secondary fragmentation products of the $[z_n + H]^+$ ions, which generate even-electron w_n^+ ions [7, 13, 24, 25]. The differenti-

Figure 5. MS³ spectrum of $[M + H]^{2+}$ with unimass resolution, where $M = \text{RVYVHPF}$. The amplitude of the resonance excitation RF voltage was 0.80 eV; helium was used as the collision gas.

Figure 6. MS³ spectra of $[M + H]^{2+}$ with unimass resolution, where (a) $M = \text{RLYVHPI}$, (b) $M = \text{RIYVHPI}$, and (c) $M = \text{RLYIHPI}$. The amplitudes of the resonance excitation RF voltage were 0.79, 0.80, and 0.80 eV, respectively; helium was used as the collision gas. The MS⁴ spectrum of $[z_4 + H]^+$ is presented in the inset.

ation of isomeric Xle is also possible from high-energy CID of protonated peptides [43] or from low-energy CID of singly and doubly charged copper-2,2'-bipyridine-peptide complexes containing isomeric leucine and isoleucine units [44–46]. In addition, the peptide radical cations obtained recently by using this electron-transfer approach has allowed Xle residues to be distinguished in isomeric tripeptides [36, 38, 43]. The latter method, however, has been demonstrated only for tripeptides having a

single Xle residue [38, 39, 44]. Figures 6a and 6b displays the spectra of isomeric heptapeptides of the form R(Xle)YVHPI containing Leu and Ile units on their respective second residues. The cleavage of the side-chain linkage between the α and β carbon atoms of certain amino acid residues immediately provides an important clue as to the amino acid composition of the peptide; e.g., the neutral side-chain radicals that are lost from Leu/Ile (56 Da), Val (42 Da), and Tyr (106 Da), respectively. The amino acid composition of

Figure 6. Continued

the peptide, but not the exact sequence information of Xle, can be deduced by subtracting the difference between the m/z values of the parent ion and the doubly charged fragment ions. In this example, $[M + H]^{2+}$ (m/z 448.5) – $[M + H - C_4H_8]^{2+}$ (m/z 420.5) = 56 Da, which identifies the cleavage as the side-chain loss of Leu/Ile. Differentiation between isomeric leucine and isoleucine residues in polypeptides is revealed by computing the m/z difference between the consecutive $[z_n + H]^+$ and w_n^+ ions, although they may exist in low abundance, arising from β and γ carbon–carbon bond cleavages of leucine and isoleucine side chains respectively. These cleavages eliminate neutral isopropyl radical and ethyl radicals respectively, because of the different connectivities of their alkyl side chains. Figure 6a displays a small peak at m/z 682.2, which is assigned as a w_6^+ ion. The difference between the m/z values of the $[z_6 + H]^+$ (m/z 724.9) and w_6^+ (m/z 682.2) ions is 43 Da, which identifies leucine as the second amino acid residue. Similarly, the presence in Figure 6b of the diagnostic w_6^+ ion at m/z 695.9 with a neutral loss of 29 Da provides a good indication that an isoleucine exists as the second amino acid residue of RIYVHPI. The proposed origin of the w_n^+ ion from the $[z_n + H]^+$ ion has been confirmed in the CID spectrum of the $[z_4 + H]^+$ ion from RLYIHPI, as presented in the inset of Figure 6c, which is in accordance with the product ions observed from high-energy CID and HECD [24, 25, 43]. Again, the presence of

$[z_4 + H]^+/w_4^+$ and $[z_6 + H]^+/w_6^+$ ions unambiguously identifies leucine and isoleucine at the second and fourth amino acid residues among the three Xle residues in the heptapeptide RLYIHPI. The lack of observable $[z_1 + H]^+$ and w_1^+ ions, however, is probably due to the intrinsic low-mass cutoff of the ion trap instrument (ca. 28% on our instrument).

Conclusions

In this study, we report the formation of hydrogen-deficient $[M + H]^{2+}$ dication by using low-energy CID and dissociating the gas-phase triply charged $[Cu^{II}(L)(M + H)]^{3+}$ ions through a one-electron-transfer mechanism. Previously, these novel $[M + H]^{2+}$ cations could be generated only through electron stripping within an accelerator mass spectrometer or through electron capture dissociation in an FTICR. Collision-induced dissociation of doubly charged $[M + H]^{2+}$ radical cations suggests that they produce similar types of fragment ions as do the corresponding radical cations obtained from HECD. The presence of $[z_n + H]^+$ and w_n^+ fragment ions is rarely observed in the spectra from low-energy tandem mass spectrometry of protonated peptides. The formation of w_n^+ ions occurs through side-chain fragmentation and allows leucine and isoleucine residues to be distinguished unambiguously in the polypeptides. This differentiation is demonstrated by the presence of peaks associated with the loss from the Xle side chains of diagnostic

neutral radicals, $\cdot\text{CH}(\text{CH}_3)_2$ (43 Da) and $\cdot\text{CH}_2\text{CH}_3$ (29 Da) and inspecting the consecutive mass differences between the $[\text{z}_n + \text{H}]^+$ and w_n^+ ions of the Xle residue. Although we have witnessed only the first few $[\text{M} + \text{H}]^{2+}$ species using this approach, the reactivities of these hydrogen-deficient radical cations, which are unencumbered by solvation, are fascinating and rich.

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