Fragmentation Chemistry of [M + Cu]+ Peptide Ions Containing an N-terminal Arginine

Sharon J. Shields, Brian K. Bluhm, and David H. Russell
Laboratory for Biological Mass Spectrometry, Department of Chemistry, Texas A&M University, College Station, Texas, USA

[M + Cu]+ peptide ions formed by matrix-assisted laser desorption/ionization from direct desorption off a copper sample stage have sufficient internal energy to undergo metastable ion dissociation in a time-of-flight mass spectrometer. On the basis of fragmentation chemistry of peptides containing an N-terminal arginine, we propose the primary Cu+ ion binding site is the N-terminal arginine with Cu+ binding to the guanidine group of arginine and the N-terminal amine. The principal decay products of [M + Cu]+ peptide ions containing an N-terminal arginine are [a_n + Cu − H]+ and [b_n + Cu − H]+ fragments. We show evidence to suggest that [a_n + Cu − H]+ fragment ions are formed by elimination of CO from [b_n + Cu − H]+ ions and by direct backbone cleavage. We conclude that Cu+ ionizes the peptide by attaching to the N-terminal arginine residue; however, fragmentation occurs remote from the Cu+ ion attachment site involving metal ion promoted deprotonation to generate a new site of protonation. That is, the fragmentation reactions of [M + Cu]+ ions can be described in terms of a “mobile proton” model. Furthermore, proline residues that are adjacent to the N-terminal arginine do not inhibit formation of [b_n + Cu − H]+ ion, whereas proline residues that are distant to the charge carrying arginine inhibit formation of [b_n + Cu − H]+ ions. An unusual fragment ion, [c_n + Cu + H]+, is also observed for peptides containing lysine, glutamine, or asparagine in close proximity to the Cu+ carrying N-terminal arginine. Mechanisms for formation of this fragment ion are also proposed. (J Am Soc Mass Spectrom 2000, 11, 626–638) © 2000 American Society for Mass Spectrometry

The binding of metal ions to functionalized organic molecules is a very active area of gas-phase ion chemistry [1, 2] and solution-phase chemistry [3]. In the area of gas-phase biomolecule chemistry, the most extensively studied species are the alkali metal ion–peptide complexes [4–14]. Much of the research in this area has focused on binding sites of alkali metal cations as inferred from the fragmentation pattern of the intact ion. The most commonly used method for such studies is tandem mass spectrometry [e.g., metastable ion (MI) and/or collision induced dissociation (CID) mass spectrometry]. In an early paper on this subject, Russell and co-workers rationalized the dissociation reactions of small peptide [M + Na]+ ions in terms of metal ion binding to the nitrogen of the amide functional group [7]. Grese and co-workers [8, 9] base their explanation of the dissociation reactions on the binding of the alkali metal ion to the deprotonated C-terminus, whereas Adams [12, 13] and Tang [4] suggest that the alkali metal cation binds to the carbonyl oxygens. Arguments concerning attachment sites of alkali metal ions to gas-phase peptides are generally based on solution-phase properties, especially those made by Adams; however, it is unclear whether a gas-phase ionic peptide in the absence of solvent and with sufficient internal energy to dissociate is accurately modeled by solution-phase concepts.

More recently, interactions of transition metal ions with model peptides have been studied by using both experimental and theoretical methods. In the specific case of Cu+, Deng and Kebärle [15] utilized equilibrium ligand exchange experiments to determine the gas-phase binding energies of small organic molecules with functional groups similar to those found on amino acid side chains. They found that the Cu+ binding energy of 1-methylimidazole is about 10 kcal/mol greater than that for an n-butyl amine–Cu+ complex. Cerda and Wesdemiotis [16] utilized the kinetic method to estimate relative Cu+ ion affinities for all 20 common amino acids assuming the amino acid acts as a monodentate ligand for Cu+ and found arginine, lysine, and histidine to have the greatest Cu+ ion affinity. More recent work suggests that some amino acids act as multidentate ligands [17, 18]. For example, the flexibility of the lysine side chain allows Cu+ to bind both the N-terminal and side chain amine groups to form a...
bidentate structure. Bidentate structures would be especially important for N-terminal lysine resulting in an increase in the Cu$^+$ binding energy.

The general trend appears to suggest that gas-phase nitrogen bases have a greater Cu$^+$ ion affinity than do sulfur bases, whereas oxygen bases have the lowest Cu$^+$ ion affinity. The trend in binding energies suggests that soft Lewis acid Cu$^+$ prefers binding to soft nitrogen bases rather than to hard oxygen bases. In aqueous solution, Cu$^+$ coordinates more strongly with the sulfur containing residues, cysteine and methionine, which are softer bases than nitrogen [19]. On the other hand, nitrogen bases such as the guanidine group of arginine and the NH$_2$ side chain of lysine are protonated in aqueous (pH < 10) solution and this greatly decreases the metal ion affinity of the nitrogen bases [3].

The fragmentation reactions of amino acid–copper ion (both Cu$^+$ and Cu$^{2+}$) complexes have been examined by several authors [20–27]. A common fragmentation reaction observed for most amino acid–Cu$^+$ complexes is loss of H$_2$CO$_2$, and it has been suggested that the product ions are formed by Cu$^+$ ion insertion reactions [21–23]. For example, Lavannant and Hoppiardi [21] and Wen et al. [22] propose Cu$^+$ insertion into the H(R)C–COOH bond of the amino acid. Although metal ion insertion into C–C bonds has been proposed for a number of metal ion–organic molecule species [1, 2], insertion reactions are unlikely for large molecules, such as peptides, that contain multiple potential Cu$^+$ ion binding sites. Metal ion insertion reactions of Cu$^{2+}$ into amino acid bonds have also been considered. For example, Gatlin and co-workers [24–27] examined the fragmentation reactions of Cu$^{2+}$ bound to amino acids and 2,2-bipyridyl ligands. They describe complexes in which the Cu$^{2+}$ is bound to the two nitrogens of the 2,2-bipyridyl ligand, the deprotonated C-terminus, and the N-terminal amine of the amino acid; however, no evidence was found to support structures corresponding to metal ion insertion into amino acid C–C bonds. Thus, it appears that both experimental and theoretical data indicates that amino acid–Cu$^+$ species are best described as ionic complexes where the peptide acts as a ligand.

Of the 20 common amino acids, arginine has the greatest gas-phase Cu$^+$ ion affinity as demonstrated by Cerda [16] and Hoyau [28]. Theoretical calculations suggest the Cu$^+$ binding energy to the imine nitrogen of guanidine is ~78 kcal mol$^{-1}$ (B3LYP level theory) [29] to ~73 kcal mol$^{-1}$ (MP2) [17, 18]. Results from fragmentation studies suggest that the Cu$^+$ binding energy to N-terminal arginines is greater than that for internal [18] and C-terminal arginine residues [30, 31]. In addition, peptides that do not contain basic residues (e.g., VGVAPG and YGGFM) do not form abundant [M + Cu$^+$] ions by matrix-assisted laser desorption/ionization (MALDI) [32] which is also consistent with the idea that Cu$^+$ ions do not strongly bind to the carbonyl oxygens of the amide group.

In this paper we report metastable ion (MI) reaction chemistry of MALDI-formed [M + Cu$^+$] peptide ions that contain an N-terminal arginine. Ensuing papers will address the chemistry of [M + Cu$^+$] peptide ions containing arginine as an internal amino acid [17] and as a C-terminal amino acid [30, 31]. For [M + Cu$^+$] peptide ions containing an N-terminal arginine, most fragment ions observed contain arginine and Cu$^+$. The only exceptions observed are for peptides that contain histidine, and in this case there are several low-abundance fragment ions that correspond to histidine complexed to Cu$^+$. Hydrogen/deuterium exchange experiments are utilized in an effort to elucidate the mechanism(s) for fragmentation of peptide–Cu$^{2+}$ complexes. Reaction mechanisms for formation of [a$_n$ + Cu – H]$^+$, [b$_n$ + Cu – H]$^+$, and [c$_n$ + Cu + H]$^+$ fragment ions are proposed. Losses of small neutral molecules from [M + Cu$^+$] precursor ions and subsequent fragment ions are also discussed.

**Experimental**

MALDI was performed on a Perseptive Biosystems Voyager Elite XL single-stage reflectron time-of-flight mass spectrometer operated in delayed ion extraction mode [33]. The individual product ion scans produced by systematically reducing the voltage applied to the reflectron were stitched together using Perseptive Biosystems software to create composite MI spectra. It is important to distinguish fragment ions formed by metastable decomposition, e.g., unimolecular dissociation of ions resulting from excess internal energy obtained during the desorption/ionization event, from product ions formed by CID. To minimize contributions of CID to the MI mass spectrum, the pressure in the drift tube was maintained at $5 \times 10^{-9}$ torr. This pressure is approximately an order of magnitude lower than that required to observe CID product ions. All peptides were purchased from Sigma (St. Louis, MO) and used without further purification. 5 pmol of analyte was deposited on the sample probe with α-cyano-4-hydroxycinnamic acid at a matrixanalyte molar ratio of 1500:1. [M + H]$^+$, [M + Cu]$^+$, and [M + H + 2Cu]$^+$ ions were generated by desorbing the sample from a copper sample stage with a 337 nm nitrogen laser [32]. The timed ion selector allows isolation and analysis of the [M + Cu$^+$] isotopic envelope and its metastable ions. Three MI spectra are acquired for each [M + Cu$^+$] peptide ion.

All fragment ions are isotopically resolved in the MI spectra revealing the 63Cu and 65Cu contribution to the isotopic envelope and its metastable ions. Three MI spectra are acquired for each [M + Cu$^+$] peptide ion.
was dissolved in MeOD under a nitrogen atmosphere. To insure full exchange, the peptides and matrix remained in their respective solvents for 12 h prior to mixing and deposition on the copper sample stage. The sample stage was transported to the instrument under nitrogen atmosphere to prevent back exchange with the ambient atmosphere.

**Results**

Figure 1a–c, respectively, contains the MI mass spectrum of \([M + Cu]^+\) ions of des-arg⁹ bradykinin, PPGFSFP, RYLGYL, and b-chain insulin fragment 22–30, RGFFYTFPKA, obtained by post source decay (PSD) focusing. Sequence ions are labeled using the nomenclature proposed by Roepstorff [34] for backbone cleavages of peptide ions. All three peptides dissociate exclusively into a-type and b-type ions which contain the N-terminal arginine and Cu⁺.

Table 1 contains data for the dissociation of deuterated des-arg⁹ bradykinin \([M + Cu]^+\) ions and the percent deuterium incorporation is reported for each ion. The \([M + Cu]^+\) ion contains 34 ± 3D₂ incorporation and 12 labile hydrogens are present in the peptide. Therefore, the maximum number of deuteriums in each fragment ion can be unambiguously determined and hypotheses can be made regarding dissociation mechanisms.

Figure 2a, b, respectively contains MI mass spectra for \([M + Cu]^+\) ions of splenopentin, RKEYV, and substance P, (RPKPQGQFLM–NH₂). Each peptide contains an N-terminal arginine, with lysine as the second and third residue, respectively. Note that each spectrum contains fragment ions that correspond to the mass of \([c_n + Cu + H]^+\) fragment ions which could be formed by cleavage of the NH–CH(R) (R = amino acid side chain) bond of lysine, e.g., ions at \(m/z\) 236.1 (Figure 2a, \([c_1 + Cu + H]^+\)) and 333.2 (Figure 2b, \([c_2 + Cu + H]^+\)). The spectrum of substance P also contains c-type fragment ions formed by bond cleavages at the glutamine residues (Q) \([c_n + Cu + H]^+\) and \([c_n + Cu + H]^+\), and a similar cleavage is observed at the asparagine (N) residue in RPNPG \([c_2 + Cu + H]^+\) in Table 2). Formation of these fragment ions suggests some interaction(s) between arginine, Cu⁺, and glutamine or asparagine residues to promote c-type bond breakage. Alternatively these fragment ions may arise by transfer of the NH₂ group of the lysine, glutamine, or asparagine to Cu⁺ through a bidentate interaction of the peptide with Cu⁺. H/D exchange experiments were performed on substance P fragment 1–4 (RPKP) \([M + Cu]^+\) ions (Figure 3) to elucidate c-type fragment ion structure. Table 3 contains data for deuterated substance P fragment 1–4 \([M + Cu]^+\) and fragment ions. To calculate the deuterium incorporation, the isotopic distribution of each nondeuterated fragment ion is normalized to the experimentally measured abundance of the monoisotopic peak. For example, the experimentally measured peak areas of the \([a_1 + Cu – H]^+\) fragment ion (\(m/z\) 191.0) are: \(m/z\) 191, 100%; 192, 10 ± 3%; 193, 41 ± 6%; 194, 5 ± 2%. In the MI spectrum of the deuterated \([M + Cu]^+\) ion, the first peak of measurable abundance occurs at \(m/z\) 194 (Figure 3) indicating incorporation of three deuterium atoms. The D₄ content is obtained from the corrected area of the \(m/z\) 195 peak (e.g., the measured peak area minus 10% of the area of the \(m/z\) 194 peak). The D₅ content is obtained by using a similar correction for the \(m/z\) 196 peak; corrected for both the \(m/z\) 195 (10% of the corrected area) and the peak at \(m/z\) 194 (41% of the corrected area). The number of deuteriums present and their percent contribution are then calculated from the corrected peak areas.

Figure 4 contains the MI spectrum of KRQHPG \([M + Cu]^+\) ions. This peptide was studied to evaluate the effect of an N-terminal lysine adjacent to an arginine residue. Note that c-type fragment ions are observed at arginine and glutamine, but not at histidine. In addition, two internal fragment ions are observed that contain glutamine and histidine, \([a_1 y_4]_2 + Cu^+\) and \([b_2 y_4] + Cu^+\), complexed to Cu⁺, indicating that histidine has a relatively large Cu⁺ ion affinity.

All MI spectra investigated for this paper have several similar characteristics. First, Cu⁺ does not dissociate from the peptide under metastable dissociation conditions as evidenced by the absence of an ion at \(m/z\) 62.9/64.9, corresponding to Cu⁺. For the peptides investigated, we find that all \([M + Cu]^+\) peptide ions yield product ions at \(m/z\) 191.0 which corresponds to an \([a_1 + Cu – H]^+\) ion for an N-terminal arginine or the monomion ion of arginine complexed to Cu⁺. Also note that there are no low mass fragment ions below \(m/z\) 191.0 except that of the low abundance monomion ions of lysine complexed to Cu⁺ (Figure 4).

**Discussion**

The dissociation of α-amino acids complexed to Cu⁺ has been investigated by several groups [21–23]. Although the ionization and mass analysis techniques differ, each group observed a neutral loss of 46 Da corresponding to loss of H₂O and CO from the amino acid–Cu⁺ complex. Conversely, in peptide–Cu⁺ complexes, we do not observe the loss of 46 Da from any \([M + Cu]^+\) or fragment ion. This observation is consistent with the idea that peptides have a multitude of functional groups that readily bind Cu⁺.

The present experiments reveal that \([M + Cu]^+\) ions of small peptides formed by MALDI from a copper surface undergo dissociation reactions that can be rationalized on the basis of competitive binding of Cu⁺ between the arginine side chain and other basic amino acid side chains. We postulate that (i) fragmentation reactions of \([M + Cu]^+\) ions are governed by relative binding energies of Cu⁺ to side chain functional groups with the most basic site acting as an anchor for Cu⁺ and (ii) dissociation pathways of peptides containing basic residues can be used to identify the binding site(s) of the Cu⁺ ion. All fragment ions of peptides containing...
FRAGMENTATION OF [M + Cu]⁺ PEPTIDE IONS

A

B

C
nium ions and internal fragment ions. For example, ions, focusing the discussion on dissociation reactions of [M + Cu]⁺ ions and Cu⁺ ion binding sites. In a subsequent paper, we will deal specifically with Cu⁺ bound to glutamine-histidine rather than Cu⁺ bound to arginine. The absence of internal fragment ions containing proline suggests that proline does not possess a strong Cu⁺ ion affinity and has less influence on formation of internal ions than generally observed for [M + Cu]⁺ ions. That is, formation of internal fragment ions from [M + Cu]⁺ ions is less dependent on structural features of the peptide and more dependent on the competitive Cu⁺ ion binding energies between basic residues. The presence of [QH + Cu⁺] internal fragment ions suggests that the Cu⁺ ion affinity for glutamine and/or histidine must also be rather large. In particular, the immonium ions of proline, arginine, and phenylalanine, and the internal fragment ions containing proline are observed as in the MI spectrum of des-arg⁹ bradykinin [M + H⁺] ions (Figure 5). Conversely, the only immonium ions formed from [M + Cu⁺] peptide ions are those of arginine (m/z 191.0) (Figures 1, 2, and 4) and lysine (m/z 163.0) (Figure 4) complexed to Cu⁺, and the only internal fragment ions observed are [(a₇y₂)₂ + Cu⁺] and [(b₅y₂)₂ + Cu⁺] from KRQHPG [M + Cu⁺] ions (Figure 4). Note also that the internal fragment ions of KRQHPG correspond to Cu⁺ bound to glutamine-histidine rather than Cu⁺ bound to arginine. The absence of internal fragment ions containing proline suggests that proline does not possess a strong Cu⁺ ion affinity and has less influence on formation of internal ions than generally observed for [M + Cu]⁺ ions. That is, formation of internal fragment ions from [M + Cu]⁺ ions is less dependent on structural features of the peptide and more dependent on the competitive Cu⁺ ion binding energies between basic residues. The presence of [QH + Cu⁺] internal fragment ions suggests that the Cu⁺ ion affinity for glutamine and/or histidine must also be rather large. In particular, the imidazole of histidine competes with the guanidine of arginine for the Cu⁺ ion as the binding energies of the two functional groups differ by approximately 8

Table 1. H/D exchange data for ions of deuterated des-arg⁹ bradykinin [M + Cu⁺] ions

<table>
<thead>
<tr>
<th>Ion</th>
<th>m/z</th>
<th>% H/D exchange</th>
<th>Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>[a₁⁺ + Cu – H/D]⁺</td>
<td>191.0* (6)ᵇ</td>
<td>13 (1)⁹</td>
<td>1 (1)⁹</td>
</tr>
<tr>
<td>[b₂⁺ + Cu – H/D]⁺</td>
<td>319</td>
<td>320</td>
<td>321</td>
</tr>
<tr>
<td>[a₃⁺ + Cu – H/D]⁺</td>
<td>388</td>
<td>389</td>
<td>390</td>
</tr>
<tr>
<td>[a₄⁺ + Cu – H/D]⁺</td>
<td>445</td>
<td>446</td>
<td>447</td>
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<tr>
<td>[b₅⁺ + Cu – H/D]⁺</td>
<td>473</td>
<td>474</td>
<td>475</td>
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<tr>
<td>[b₆⁺ + Cu – H/D]⁺</td>
<td>593</td>
<td>594</td>
<td>595</td>
</tr>
<tr>
<td>[b₇⁺ + Cu – H/D]⁺</td>
<td>621</td>
<td>622</td>
<td>623</td>
</tr>
<tr>
<td>[b₈⁺ + Cu – H/D]⁺</td>
<td>682</td>
<td>683</td>
<td>684</td>
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<tr>
<td>[b₉⁺ + Cu – H/D]⁺</td>
<td>691</td>
<td>692</td>
<td>693</td>
</tr>
<tr>
<td>[b₁₀⁺ + Cu – H/D]⁺</td>
<td>711</td>
<td>712</td>
<td>713</td>
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<tr>
<td>[M + Cu⁺]⁺</td>
<td>974</td>
<td>975</td>
<td>976</td>
</tr>
<tr>
<td>[M + H⁺]⁺</td>
<td>22 (3)</td>
<td>32 (4)</td>
<td>36 (4)</td>
</tr>
</tbody>
</table>

*Mass of the nondeuterated ion.
*bNumber of exchangeable hydrogens before elimination of hydrogen.
⁹Average percent H/D exchange from three MI spectra and associated error for each ion.

Figure 1. (a) Metastable ion (MI) spectrum of des-arg⁹ bradykinin, RPPGFSPF, [M + Cu⁺] ions. Note the presence of [b₅ + Cu – H/D]⁺, [b₆ + Cu – H/D]⁺, and [a₇ + Cu – H/D]⁺ and the absence of [b₇ + Cu – H/D]⁺, [b₈ + Cu – H/D]⁺, and [b₉ + Cu – H/D]⁺ fragment ions. (b) MI spectrum of RYLGYL [M + Cu⁺] ions. A complete series of [b₅ + Cu – H/D]⁺ and [a₇ + Cu – H/D]⁺ fragment ions is observed. (c) MI spectrum of B-chain insulin fragment 22–30, RGFFYTPKA, [M + Cu⁺] ions. A complete series of [b₅ + Cu – H/D]⁺ and [a₇ + Cu – H/D]⁺ fragment ions is observed with the exception of [b₇ + Cu – H/D]⁺ and [a₇ + Cu – H/D]⁺ fragment ions which would be cleavages of proline.
kcal/mol [17, 18], with arginine having the greater Cu\(^+\) ion affinity.

Neutral Losses from [M + Cu\(^+\)]\(^+\) and Fragment Ions

Peptides containing serine, threonine, glutamic acid, or aspartic acid often exhibit a neutral loss of H\(_2\)O from the [M + Cu\(^+\)]\(^+\) ions and its product ions. It is generally assumed that H\(_2\)O is lost from the amino acid side chain. For example, the peptide ions shown in Figures 1a, c, and 2a, lose H\(_2\)O from fragment ions containing serine ([b\(_6\) + Cu – H – H\(_2\)O])\(^+\), threonine ([b\(_6\) + Cu – H – H\(_2\)O])\(^+\), and glutamic acid ([b\(_3\) + Cu – H – H\(_2\)O])\(^+\), respectively. Serine- and threonine-containing peptides can also lose 30 and 44 Da, respect-

<table>
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<tr>
<th>Fragment ion</th>
<th>Observed mass</th>
<th>% MI current</th>
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<tbody>
<tr>
<td>[a(_1) + Cu – H](^+)</td>
<td>190.998</td>
<td>2 (1)*</td>
</tr>
<tr>
<td>[a(_2) + Cu – H](^+)</td>
<td>288.060</td>
<td>5 (1)</td>
</tr>
<tr>
<td>[b(_2) + Cu – H](^+)</td>
<td>316.039</td>
<td>5 (1)</td>
</tr>
<tr>
<td>[c(_2) + Cu – H](^+)</td>
<td>333.085</td>
<td>4 (1)</td>
</tr>
<tr>
<td>[a(_3) + Cu – H – 17](^+)</td>
<td>385.063</td>
<td>4 (1)</td>
</tr>
<tr>
<td>[a(_3) + Cu – H](^+)</td>
<td>402.080</td>
<td>8 (2)</td>
</tr>
<tr>
<td>[b(_3) + Cu – H – 18](^+)</td>
<td>412.107</td>
<td>8 (1)</td>
</tr>
<tr>
<td>[b(_3) + Cu – H](^+)</td>
<td>430.081</td>
<td>42 (4)</td>
</tr>
<tr>
<td>[M + Cu – 42](^+)</td>
<td>560.112</td>
<td>3 (1)</td>
</tr>
<tr>
<td>[M + Cu](^+)</td>
<td>602.213</td>
<td></td>
</tr>
</tbody>
</table>

*Error associated with integration of the monoisotopic peak of each fragment ion from three MI spectra.
tively, where 30 Da corresponds to formaldehyde (CH$_2$O) loss from the serine side chain and 44 Da corresponds to acetaldehyde (CH$_3$COH) loss from the threonine side chain. The MI spectrum of RGFFYPKRA [M + Cu]$^+$ ions (Figure 1c) contains a fragment ion (m/z 1104.56) that corresponds to loss of 44 Da from the [M + Cu]$^+$ ion and loss of 44 Da is also observed from the [b$_6$ + Cu - H]$^+$ fragment ion. Similar losses of neutrals from side chains of serine and threonine have been reported for anionic peptide complexes [38–40]. Although our studies are limited to cations, the reaction mechanism for neutral loss of 30 and 44 Da is probably similar to that proposed by Adams and co-workers [38].

Of particular interest for [M + Cu]$^+$ ions investigated in this study is the loss of 17 and 42 Da from peptides containing multiple basic amino acids. The MI spectra for the peptide ions shown in Figure 1 do not contain ion signals for loss of 17 or 42 Da, but MI spectra shown in Figures 2 and 3 both contain ion signals for loss of 17 and 42 Da from [M + Cu]$^+$ and fragment ions containing two basic amino acids. Loss of 17 Da is assigned to loss of NH$_3$ and most likely originates from lysine and/or arginine [41], whereas loss of 42 is assigned to loss of HN=C=NH from the guanidine group of arginine [42]. We find it interesting that HN=C=NH is lost from the guanidine group because the imine nitrogen of the guanidine group has the highest Cu$^+$ binding energy of all amino acid functionalities [17, 18, 29]. Note that loss of HN=C=NH only occurs in those cases where amino acids having NH$_2$ side chains and/or histidine are in close proximity to the N-terminal arginine. We rationalize the dependence of HN=C=NH loss on nearby NH$_2$ groups in terms of

Table 3. H/D exchange data for ions of deuterated substance P fragment 1–4 [M + Cu]$^+$ ions

<table>
<thead>
<tr>
<th>Ion</th>
<th>m/z</th>
<th>% H/D exchange</th>
<th>m/z</th>
<th>% H/D exchange</th>
<th>m/z</th>
<th>% H/D exchange</th>
<th>m/z</th>
<th>% H/D exchange</th>
</tr>
</thead>
<tbody>
<tr>
<td>[a$_1$ + Cu - H]$^+$</td>
<td>193 194 195 196 197</td>
<td>2 (1)$^b$</td>
<td>16 (2)$^c$</td>
<td>34 (3)$^c$</td>
<td>46 (7)$^c$</td>
<td>2 (2)$^c$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[a$_2$ + Cu - H]$^+$</td>
<td>290 291 292 293 294</td>
<td>318 319 320 321 322</td>
<td>4 (1)</td>
<td>17 (6)</td>
<td>32 (2)</td>
<td>46 (4)</td>
<td>1 (1)</td>
<td></td>
</tr>
<tr>
<td>[b$_2$ + Cu - H]$^+$</td>
<td>337 338 339 340 341</td>
<td>333/1$^*$ (7)$^b$</td>
<td>7 (1)</td>
<td>10 (3)</td>
<td>29 (3)</td>
<td>31 (3)</td>
<td>23 (4)</td>
<td></td>
</tr>
<tr>
<td>[c$_2$ + Cu - H]$^+$</td>
<td>449 450 451 452 453</td>
<td>444.2$^*$ (9)$^b$</td>
<td>3 (1)</td>
<td>16 (3)</td>
<td>40 (1)</td>
<td>40 (4)</td>
<td>1 (1)</td>
<td></td>
</tr>
<tr>
<td>[b$_3$ + Cu - H + 18]$^+$</td>
<td>469 470 471 472 473</td>
<td>462.2$^*$ (9)$^b$</td>
<td>8 (1)</td>
<td>20 (3)</td>
<td>35 (4)</td>
<td>34 (7)</td>
<td>3 (2)</td>
<td></td>
</tr>
<tr>
<td>[M + Cu - 42]$^+$</td>
<td>522 523 524 525 526</td>
<td>517.2$^*$ (10)$^b$</td>
<td>5 (2)</td>
<td>24 (2)</td>
<td>30 (2)</td>
<td>40 (5)</td>
<td>1 (1)</td>
<td></td>
</tr>
<tr>
<td>[M + Cu]$^+$</td>
<td>565 566 567 568 569</td>
<td>559.2$^*$ (10)$^b$</td>
<td>2 (1)</td>
<td>7 (1)</td>
<td>19 (1)</td>
<td>39 (1)</td>
<td>33 (4)</td>
<td></td>
</tr>
</tbody>
</table>

*Mass of the nondeuterated ion.

$^b$Number of exchangeable hydrogens before elimination or addition of hydrogen.

$^c$Average percent H/D exchange from three MI spectra and associated percent error for each ion.

Figure 3. MI spectrum of deuterated substance P fragment 1–4, RPKP, [M + Cu]$^+$ ions.
the lability of binding sites that are available to Cu$^+$. As the number of ligands increases, the strength of individual ligand–Cu$^+$ interactions decreases, opening up new dissociation channels. The idea that strongly bound Cu$^+$ ligands can be eliminated when additional ligands are bound to Cu$^+$ is supported by both theoretical and experimental studies of Cu$^+$–NH$_3$ clusters. Bauschlicher et al. [43] and Walker et al. [44] found that the first and second NH$_3$ ligands have similar Cu$^+$ binding energies; however, addition of a third and fourth NH$_3$ ligand causes a decrease in the individual ligand binding energies due to “increased ligand–ligand repulsion [43].” The results on NH$_3$–Cu$^+$ clusters supports the idea that multiple ligands bound to Cu$^+$ decreases the bond strength and permits loss of neutral fragments from the [peptide + Cu]$^+$ ionic complex.

**Formation of $[b_n + Cu - H]^+$ Fragment Ions**

Fragment ions such as $[b_n + Cu - H]^+$ are formed by cleavage of peptide backbone amide bonds with Cu$^+$ being retained by the N-terminal fragment. A plausible mechanism for formation of $[b_n + Cu - H]^+$ ions is amide bond dissociation with hydrogen transfer from the α-carbon to the neutral leaving group as proposed by Adams and co-workers (Scheme 1a) for formation of $[b_n + Cat - H]^+$, where Cat corresponds to an alkali metal ion [13]. Alternatively, Scheme 1b shows a mechanism that is similar to that proposed by Yalcin and
ions because both amino acids contain an amide hydrogen. However, proline appears to inhibit formation of \( b_2 + Cu - H \)\(^+ \) fragment ions at proline in terms of hydrogen transfer occurring from the amide nitrogen rather than from the \( \alpha \)-carbon because proline does not contain an amide hydrogen to transfer. However, neither Scheme 1b nor Scheme 1c adequately describe formation of \( b_2 + Cu - H \)\(^+ \) from des-arg\(^9\) bradykinin and substance P as there is no amide hydrogen present to form this ion.

Results from hydrogen/deuterium exchange experiments provide additional insight into the mechanism for formation of \( b_2 + Cu - H \)\(^+ \) fragment ions. For example, des-arg\(^9\) bradykinin (RPPGFSPF) contains prolines at positions 2, 3, and 7. Note that proline does not inhibit formation of \( b_2 + Cu - H \)\(^+ \), whereas the \( b_3 + Cu - H \)\(^+ \) and \( b_7 + Cu - H \)\(^+ \) fragment ions are not observed. The \( b_2 + Cu - H \)\(^+ \) fragment ion has a nondeuterated mass of 316.1 Da with six labile hydrogens in the H-Arg-Pro section of its precursor ion. Note that 18 ± 5% of fragment the ions contain three deuteriums, 33 ± 5% contain four deuteriums, 47 ± 1% contain five deuteriums, but there is no evidence for six exchanged hydrogens (Table 1). Consequently, we conclude that the \( b_2 + Cu - H \)\(^+ \) fragment ion corresponds to \( b_2 + Cu - D \)\(^+ \), e.g., a deuterium is transferred to the neutral fragment. All other b-type ions of des-arg\(^9\) bradykinin [M + Cu]\(^+ \) ions correspond to \( b_n + Cu - D \)\(^+ \). The deuterium distribution in the \( b_2 + Cu - D \)\(^+ \) fragment ion is not consistent with a mechanism (Scheme 1a) involving hydrogen atom transfer from the \( \alpha \)-carbon atom. The mechanisms shown in Scheme 1b, c, are more reasonable because the hydrogen atom transferred is an exchangeable one. Scheme 1b, c, however, do not explain the presence of \( b_2 + Cu - H \)\(^+ \) in des-arg\(^9\) bradykinin and substance P. In this case, hydrogen transfer comes from the N-terminal side of the cleavage and is also an exchangeable hydrogen. Scheme 2 illustrates formation of \( b_2 + Cu - H \)\(^+ \) ions via proton transfer from the N-terminus to the amide nitrogen of the third residue. The N-terminal nitrogen is then a nucleophile, which attacks
the carbonyl of proline, to form a six-membered ring, accompanied by amide bond cleavages between the second and third residues. The initial proton transfer is promoted by Cu$^+$ bound to both the guanidine of arginine and the N-terminal amine. The protonated amide nitrogen in Schemes 1b and 2 induces weakening of the amide bond to promote b-type ion formation. Such a reaction mechanism is supported by ab initio calculations. Somogyi and co-workers [48, 49] used ab initio calculations to predict bond strengths and bond orders based on different sites of protonation. They found that protonation of amide nitrogens weakens the amide bond, whereas protonation at carbonyl oxygens strengthens the amide bond. Therefore, we conclude that Cu$^+$ ionizes the peptide but fragmentation reactions occur remote from the Cu$^+$ charge; however, it is incorrect to consider fragmentation reactions of [M + Cu$^+$] ions as examples of remote-site fragmentation because fragmentation reactions leading to formation of [b$_n$ + Cu − H]$^+$ involves a metal ion promoted deprotonation reaction to generate a new site of protonation (Scheme 1b). It is also interesting to consider similarities between the mechanism shown in Scheme 2 and the mobile proton model [36] (vide infra).

A combination of Schemes 1b, c and 2 can be used to explain suppression of [b$_n$ + Cu − H]$^+$ by proline (for $n > 2$) for N-terminal arginine containing peptides. For example, Schemes 1b, c suggests that the absence of hydrogen on the amide nitrogen prevents [b$_n$ + Cu − H]$^+$ fragment ion formation. On the other hand, Scheme 2 suggests hydrogen transfer is possible from the N-terminal amine group to proline. Note, this b-type ion only occurs when proline is adjacent to an N-terminal arginine which can be explained in terms of arginine, anchoring the Cu$^+$ ion, demonstrating that the proline induced b-type fragment ion is charge dependent, whereas a proline that is not adjacent to the charge carrying arginine inhibits formation of [b$_n$ + Cu − H]$^+$ fragment ions.

Scheme 1b, c predicts formation of cyclic [b$_n$ + Cu − H]$^+$ fragment ions. We would expect a cyclic [b$_n$ + Cu − H]$^+$ ion shown in Scheme 1b to be more stable than the one shown in Scheme 1c, due to steric strain in the three-membered ring as compared to a five-membered ring. In addition, proton transfer from the amide nitrogen to the neutral leaving group is more reasonable than proton transfer from the α-carbon atom. For example, Cu$^+$ is anchored by the N-terminal arginine, thus proton transfer from the amide nitrogen can be rationalized in terms of the mobile proton model [36]. The mobile proton is important because it ionizes the amide nitrogen, thereby weakening the amide bond to yield [b$_n$ + Cu − H]$^+$ fragment ions.

**Formation of [a$_n$ + Cu − H]$^+$ Fragment Ions**

There is some question as to whether a-type fragment ions are formed by simple bond cleavage of the peptide backbone or by elimination of CO from b-type fragment ions [45, 46]. We find evidence that at least in some cases [a$_n$ + Cu − H]$^+$ fragment ions are formed by direct cleavage of the α-carbon–carbonyl bond. Hu et al. [50] also suggested that [a$_n$ − H]$^+$ fragment ions of [M + Metal(II) − H]$^+$ peptide ions are formed by direct cleavage of the H(R)C–CO bond.

If a-type fragment ions are formed by elimination of CO from b-type ions, then a-type fragment ions should not be observed in the absence of the corresponding b-type ion. Thus, glycine should not inhibit formation of [a$_n$ + Cu − H]$^+$, whereas proline should inhibit formation of [a$_n$ + Cu − H]$^+$ when $n > 2$. The MI spectra contained in Figures 1a–c and 2b clearly show that glycine does not prevent formation of [a$_n$ + Cu − H]$^+$ ions. Proline does not inhibit formation of [a$_2$ + Cu − H]$^+$ ions when it is adjacent to the N-terminal arginine; however, proline does inhibit formation of [a$_n$ + Cu − H]$^+$ ions when proline is the $n = 2$ amino acid. The [a$_3$ + Cu − H]$^+$ fragment ion on from des-arg$^9$ bradykinin [M + Cu$^+$] ions is an anomaly because it is observed in the absence of the [b$_4$ + Cu − H]$^+$ fragment ion. All other a-type ions are observed in conjunction with the corresponding b-type ion except that the immonium ion of arginine, labeled [a$_1$ + Cu − C − H]$^+$, is observed in the absence of [b$_1$ + Cu − H]$^+$. Schemes 1b, c suggests formation of [b$_3$ + Cu − H]$^+$ from the des-arg$^9$ bradykinin [M + Cu$^+$] ions is not observed because there is no hydrogen on the proline amide nitrogen for proton transfer. Formation of [a$_n$ + Cu − H]$^+$ in the absence of [b$_4$ + Cu − H]$^+$ in des-arg$^9$ bradykinin suggests that the a-type ions result from a direct cleavage of the the H(R)C–CO bond.

Results from hydrogen/deuterium exchange experiments also provide interesting details about formation of [a$_n$ + Cu − H]$^+$ fragment ions. The [a$_n$ + Cu − H]$^+$ fragment ions formed from des-arg$^9$ bradykinin [M + Cu$^+$] ions suggest that an exchangeable hydrogen is transferred to the neutral leaving group. Because the corresponding deuterated b-type ions are also [b$_n$ + Cu − D]$^+$, this suggests that [a$_n$ + Cu − H]$^+$ (n = 1, 4, 5, 6) fragment ions could be formed by loss of CO from b-type ions. Thus, the cyclic b-type ion, shown in Scheme 1b, c, undergoes ring opening followed by CO elimination (Scheme 3a, b).

Alternatively, [a$_n$ + Cu − H]$^+$ ions could be formed by direct cleavage of the H(R)C–CO bond with hydrogen transfer from the amide nitrogen as shown in Scheme 3c. A similar mechanism to Scheme 3c was proposed by Hu and co-workers for divalent transition metal ion–peptide complexes [50], but, it was ruled out because they primarily observed [a$_n$ + Metal$^{12+}$ − H]$^+$ ions at amino acids with aromatic side chains, viz., tyrosine and phenylalanine. Formation of [a$_n$ + Cu − H]$^+$ ions does not appear to depend upon the type of side chain because it is observed in conjunction with all amino acids with the occasional exception of proline. Results from H/D exchange experiments on des-arg$^9$ bradykinin do not rule out the mechanisms shown in Schemes 4 and 5; however, there are additional obser-
vations that should be noted. The \([a_3 + Cu - H]^+\) fragment ion is observed for des-arg bradykinin \([M + Cu]^+\) ions even though the \([b_3 + Cu - H]^+\) ion is not observed. There are six exchangeable hydrogens in the \([a_3 + Cu - H]^+\) fragment ion, and the deuterated fragment ion maintains six deuteriums (Table 1). In this case, hydrogen transfer from amide nitrogens (as observed for other a-type fragment ions in other cases) is not possible from the two prolines. Instead the hydrogen atom transferred originates from the backbone \(\alpha\)-carbon or \(\beta\)-carbon from the side chain. This clearly illustrates that the \([a_3 + Cu - H]^+\) fragment ion is a direct backbone cleavage and is not formed via a b-type ion which raises the possibility that all \([a_n + Cu - H]^+\) fragment ions are a result of \(H(R)C–CO\) backbone cleavage. It may be possible to elucidate this issue further by MS\(_n^\nu\) experiments, but we have not made such measurements.

**Formation of \([c_n + Cu + H]^+\) Fragment Ions**

MALDI-formed \([M + H]^+\) peptide ions do not often undergo metastable ion fragmentation to produce c-type fragment ions. Figures 2 and 3 contain MI spectra of three \([M + Cu]^+\) peptide ions, splenopentin (RKEVY), substance P (RPKPQQFFGLM–NH\(_2\)), and KRQHPG, which do contain abundant c-type fragment ions. Note that RKEVY decomposes to form \([c_1 + Cu + H]^+\) ions, substance P forms \([c_2 + Cu + H]^+\), \([c_4 + Cu + H]^+,\) and \([c_5 + Cu + H]^+\) ions, and KRQHPG forms \([c_1 + Cu + H]^+\) and \([c_2 + Cu + H]^+\) ions. Note also that the model peptide RPNPG, which does contain asparagine, also forms a \([c_2 + Cu + H]^+\) fragment ion. These fragment ions can be rationalized by \(HN–CH(R)\) bond cleavage with hydrogen transfer to the N-terminal fragment ion. Observation of \([c_i + Cu + H]^+\) fragment ions is dependent on the presence of lysine, glutamine, asparagine, and arginine (amino acids containing \(NH_2\) side chain functional groups) in close proximity to the N-terminal arginine or lysine. In addition, when the \(NH_2\) side chain functional group is distant from the N-terminal arginine or lysine, \([c_i + Cu + H]^+\) ions are not observed. For example, no \([c_6 + Cu + H]^+\) fragment ion is formed in the decomposition of RGFFYT-PKA \([M + Cu]^+\) ions (Figure 1c), suggesting \(Cu^+\) is primarily solvated by ligands near the charge site forming a multidentate structure. The amino acid specificity of \([c_i + Cu + H]^+\) ion formation leads us to question the original assignment of these fragment ions. A possible reassignment of the ions is \([b_n + Cu - H + NH_2]^+\) formed by cleavage of an amide bond accompa-
nied by intramolecular transfer of NH₃ to the Cu⁺-containing ion.

The proposed assignment as \([c_n + Cu + H]^+\) (or \([b_n + Cu + H + NH3]^+\)) fragment ions was investigated by deuterium labeling of substance P fragment 1–4 (RPKP) (Figure 3 and Table 3). The MI spectrum of RPKP \([M + Cu]^+\) ions contains an ion signal \((m/z \ 333.1)\), that could be assigned as \([c_2 + Cu + H]^+\) or \([b_2 + Cu + H + NH3]^+\) fragment ions, where the ion incorporates a maximum of eight deuteriums when formed from its fully deuterated precursor ion. However, there are seven labile hydrogens in the H-Arg-Pro-NH₃+ section. This indicates that the hydrogen transferred is an exchangeable hydrogen. Because the fourth amino acid of substance P fragment 1–4 is proline, which does not possess an amide hydrogen, we can rule out the possibility that deuterium is transferred from this nitrogen. The only remaining sources of labile hydrogens that could be transferred are the ε-NH₂ of lysine or the carboxylic acid of the C-terminus. Because c-type ions only occur at amino acids containing side chain NH₂ functional groups, the hydrogen transferred to the amide nitrogen upon HN–CH(R) bond cleavage is postulated (Scheme 4) to originate from the side chain. However, there are several problems with this mechanism, with the most important involving the proton transfer to the amide nitrogen. As discussed earlier, protonation of an amide nitrogen weakens the amide bond causing amide bond cleavage (b-type ions) and not cleavage of the adjacent backbone HN–CH(R) bond (c-type ions) suggesting that the ions formed are not due to c-type bond cleavages.

Scheme 5 illustrates formation of the \([b_2 + Cu - H + NH3]^+\) ion from RPKP \([M + Cu]^+\) ions. In his mechanism, a proton from the N-terminus is transferred to the NH₂ group on the lysine side chain. Cleavage of the side chain leaves NH₂ bound to Cu⁺, whereas cleavage of the amide bond forms a \([b_2 + Cu - H + NH3]^+\) ion. When lysine is distant from the N-terminal arginine as in RGFFYTPKA, Cu⁺ is solvated by functional groups closer to the arginine, limiting coordination of lysine side chain and preventing formation of \([b_8 + Cu - H + NH3]^+\). The mechanism suggests that histidine does not promote formation of a \([b_n + Cu - H + NH3]^+\) fragment ion because of the lack of an NH₂ functional group on the side chain, even though the imidazole nitrogen of histidine has a large Cu⁺ ion affinity of \(\sim 70 \text{ kcal mol}^{-1}\). Note that the MI spectrum of KRQHPG \([M + Cu]^+\) ions (Figure 4) does not contain a \([c_2 + Cu + H]^+\) ion at histidine which also suggests that “c-type” ions may indeed be \([b_2 + Cu - H + NH3]^+\) fragment ions. H/D exchange does not reveal information concerning the structure of this ion because each fragment ion in Schemes 4 and 5 contains the same number of exchangeable hydrogens. However, the H/D exchange data suggests that the ions are \([c_n + Cu + D]^+\) or \([b_n + Cu + D - ND3]^+\) and opens a debate as to the structure of the fragment ion.

Conclusions

This paper addresses the influence of an N-terminal arginine on fragmentation reactions and gas-phase structures of peptide–Cu⁺ complexes. The guanidine functionality of arginine anchors the Cu⁺ ion allowing peptide chelation around the metal ion. Structures of gas-phase \([M + Cu]^+\) ions differ dramatically from \([M + H]^+\) ions of the same peptide which was demonstrated by the lack of internal fragment ions of proline-containing peptides as well as the lack of immonium ions. The difference lies in the immobilization of the Cu⁺ ion in \([M + Cu]^+\) ions, whereas the H⁺ is mobilized along the backbone and side chains in \([M + H]^+\) ions [37]. We propose several specific mechanisms for formation of \([a_n + Cu - H]^+\), \([b_n + Cu - H]^+\), and \([c_n + Cu + H]^+\) fragment ions. With the Cu⁺ ion anchored at the N-terminal arginine, \([b_n + Cu - H]^+\) fragment ions are formed via a mobile proton that is transferred from the N-terminus to other amide nitrogens along the peptide backbone. Although Cu⁺ may be anchored remote from the site of cleavage, the mobile proton directs fragmentation. From \([M + H]^+\) ions the \(a_n\) fragment ions are thought to be a decay product of \(b_n\) fragment ions by elimination of CO [46, 47]. However, we have evidence that suggests \([a_n + Cu - H]^+\) fragment ions are the result of a direct cleavage of the (R)HC–CO backbone bond, not a product of the \([b_n + Cu - H]^+\) fragment ion.

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