Investigation of the Presence of *b* Ions in Electron Capture Dissociation Mass Spectra

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Previously, we have indicated (Cooper, H.J., et al. *Int. J. Mass Spectrom.*, **2003**, 228, 723–728) that electron capture dissociation (ECD) of the doubly protonated peptides, Leu₄-Sar-Leu₃-Lys-OH, Leu₄-Ala-Leu₃-Lys-OH, Gly₄-Sar-Gly₃-Lys-NH₂, and Gly₃-Pro-Sar-Gly₃-Lys-NH₂, results in abundant *b* ions, which derive from fragmentation of backbone amide bonds, a nonstandard fragmentation channel in ECD. The instrumental conditions were such that the possibility that collision-induced dissociation processes were contributing to the observed spectra was eliminated. In a separate study (Fung, Y.M.E., et al. *Eur. J. Mass Spectrom.*, **2004**, 10, 449–457. ECD of peptides Arg-(Gly)_n-Xxx-(Gly)_n-Arg, where Xxx is the amino acid of interest, did not result in *b* ions. The variation in ECD observed for strikingly similar peptides suggests that the nature of the charge carrier (Arg or Lys) is instrumental in governing the fragmentation channels. Here, we describe the ECD behavior of a suite of model peptides designed such that the nature and position of the charge carrier could be probed. The results suggest that the presence of *b* ions in ECD spectra is a consequence of both charge carrier and peptide structure. Possible mechanisms for the formation of *b* ions following electron capture are discussed. (*J Am Soc Mass Spectrom* 2005, 16, 1932–1940) © 2005 American Society for Mass Spectrometry

The advent of electron capture dissociation (ECD) [1] Fourier transform ion cyclotron resonance (FT-ICR) [2] mass spectrometry constituted a significant advance in biomolecular analysis. The technique involves irradiation of trapped ions with low-energy electrons and results in fragmentation of the precursor ions. ECD displays a number of features unique among tandem mass spectrometry (MS/MS) techniques: ECD results in cleavage of peptide backbone N—C α bonds to produce *c* and *z* $\cdot$ (or *c* $\cdot$ and *z*) fragment ions, whereas traditional MS/MS techniques cleave peptide backbone amide bonds and form *b* and *y* ions [3, 4]; ECD results in preferential cleavage of disulfide bonds [5]; ECD peptide (protein) backbone fragments retain posttranslational modifications [6], for example, phosphorylation [7, 8], glycosylation [9, 10], and ubiquitination [11]. Other fragmentation channels observed following electron capture by peptides and proteins include hydrogen atom loss [12], the production of *a* $\cdot$ and *y* ions [5], and cleavage within amino acid side chains [13].

The mechanism by which ECD of N—C α bonds in peptides and proteins occurs has been a matter of considerable debate [1, 5, 14–23]. One proposed mechanism [1] involves capture of an electron by a charged site in the ion, for example, a protonated lysine or

arginine residue, followed by H $\cdot$ transfer to (or recapture by) an amide carbonyl resulting in an aminoketyl radical. The radical subsequently dissociates via cleavage of the N—C α bond. Originally, it was suggested that *c/z* $\cdot$ fragment ion formation proceeded faster than vibrational energy redistribution, and that the process was nonergodic [1]. Recent work by Tureček suggests that N—C α bond dissociation is particularly facile in thermalized radicals and radical cations, that is, nonergodicity does not apply [21]. McLafferty and coworkers [24] showed that infrared multiphoton dissociation of thermalized [M + 12H]¹¹⁺ ions results in the dominant loss of H $\cdot$ atoms in marked contrast to the *c/z* ions produced following electron capture by [M + 12H]¹²⁺ ions to form [M + 12H]¹¹⁺ radical ions. These researchers concluded that ECD was nonergodic and the mechanism involves electron capture in a high-*n* Rydberg state followed by cooling to a dissociative electronic state. A direct mechanism has been suggested in which electron capture at C_{OH}⁺ is accompanied by cleavage of the N—C α bond. Uggerud and coworkers [25, 26] have shown theoretically and experimentally that both hydrogen transfer and direct processes play a role in ECD. Recently, Syrstad and Tureček [27] have proposed a mechanism for ECD that explains the low selectivity of N—C α cleavage and applies equally to peptide ions whose charge carriers are not protons. Either electron capture in a ground electronic state results in hydrogen atom transfer from the ammonium group to the amide carbonyl to which it is solvated or electron capture in

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excited electronic states results in formation of an amide suberbse that abstracts a proton from an accessible site.

Previous work in our laboratory showed that ECD of the doubly protonated peptides Leu₄-Sar-Leu₃-Lys-OH, Leu₄-Ala-Leu₃-Lys-OH, Gly₄-Sar-Gly₃-Lys-NH₂, and Gly₃-Pro-Sar-Gly₃-Lys-NH₂, results in extensive secondary fragmentation [28]. The *w* ions resulting from dissociation of *z*[•] ions were observed. (The formation of *w* ions has also been observed in hot ECD [29], a related technique that uses higher energy electrons than ECD). In addition to *w* ions, entire and, in some cases multiple cleavages of amino acid side chains from backbone fragments were observed. Extensive water loss from backbone fragments was observed for the glycine-rich peptides. For Leu₄-Ala-Leu₃-Lys-OH, the preferred fragmentation channel was cleavage of the amide bond to produce *b*₇ and *b*₈ ions. ECD of the peptides Leu₄-Sar-Leu₃-Lys-OH, Gly₄-Sar-Gly₃-Lys-NH₂, and Gly₃-Pro-Sar-Gly₃-Lys-NH₂ also revealed the presence of abundant *b* ions. Fragmentation of the peptide amide bond to form *b* ions is a nonstandard ECD channel. Occasionally, *b* ions are observed in ECD spectra and their presence has been ascribed to collision-induced dissociation (CID) processes [30, 31]. For example, we have previously observed fragments deriving from facile bond cleavages, e.g., N-terminal to proline. Those fragments were observed in experiments in which stored waveform inverse Fourier transform (SWIFT) isolation and long irradiation times (30 s) were used. Instrumental conditions (quadrupole isolation and millisecond electron irradiation period) in both of our previous study [28] and the current work were such that the possibility that CID processes were contributing to the observed spectra was eliminated.

Recently, Chan and coworkers published a comparative study of CID and ECD of some model peptides [32]. ECD of the peptides Arg-Gly₃-Trp-Gly₃-Arg, Arg-Gly₃-Asp-Gly₃-Arg, and Arg-Gly₃-Glu-Gly₃-Arg resulted in full sequence coverage of *c* and *z*[•] fragment ions. No *b* ions were observed. The variation in ECD observed for strikingly similar peptides suggests that the nature of the charge carrier (Arg or Lys) may be instrumental in governing the fragmentation channels. Further evidence to support this hypothesis arises from the ECD spectrum obtained from Lys-Gly₃-Trp-Gly₃-Lys, in which the *b*₈ ion is observed [32]. Precursor ion selection was achieved by correlated sweep ejection (COSE). Electron irradiation time was 30–100 ms.

Iavorone et al. [33] studied the effect of charge state and cationizing agent on the ECD of the peptide Ac-(Ala-Lys-Ala-Ala-Lys)₃-Ala-NH₂. ECD of the [M + 2H]²⁺ and [M + 3H]³⁺ ions did not result in *b* ions. However, *b* ions were observed in the ECD spectra of the 4+ and 5+ parent ions. Whether these *b* ions are the result of ECD or CID processes is unclear: ion trapping in the ICR cell was enhanced by use of a nitrogen pulse. Selection of the precursor ion was achieved by COSE. Electron irradiation times varied between 500 ms and 5 s.

Here, we investigate the effect of the nature and position of the charge carrier on the ECD spectra of a suite of model peptides: Leu₄-Pro-Leu₄-Lys, Leu₄-Pro-Leu₄-Arg, Gly₄-Pro-Gly₄-Lys, Gly₄-Pro-Gly₄-Arg, Lys-Leu₄-Pro-Leu₄, and Arg-Leu₄-Pro-Leu₄. Peptide ions were doubly charged, that is, the charged sites were the terminal amino group and the side chain of the basic residue, either Lys or Arg. The peptides were designed such that the position of the basic residue, N- or C-terminal, could be investigated. The effect of amino acid side chain, Gly or Leu, was also examined. The phenomenon of proline *c/z* cleavage observed previously [28] was investigated by inclusion of Pro residues in the peptides. Substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH₂) and its all-Lys (Lys-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH₂) and all-Arg (Arg-Pro-Arg-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH₂) counterparts were also investigated. The results suggest that electron capture-mediated production of *b* fragments is dependent not only on the charge carrier, but also on the peptide ion structure. Possible mechanisms for dissociation of peptide ions to *b* fragments following electron capture are discussed.

Experimental Methods

Samples

Substance P was purchased from Sigma (St. Louis, MO) and used without further purification. Peptides Leu₄-Pro-Leu₄-Lys, Leu₄-Pro-Leu₄-Arg, Gly₄-Pro-Gly₄-Lys, Gly₄-Pro-Gly₄-Arg, Lys-Leu₄-Pro-Leu₄, Arg-Leu₄-Pro-Leu₄, Lys-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH₂, and Arg-Pro-Arg-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH₂ were synthesized by American Peptide Co., Inc., (Sunnyvale, CA) and used without further purification. The peptides were microelectrosprayed at 10 μM from solutions of 1:1 water/acetonitrile (J. T. Baker, Philipsburg, NJ), 1% formic acid (Aldrich, Milwaukee, WI).

ECD FT-ICR Mass Spectrometry

Experiments were performed on a homebuilt, passively shielded, 9.4 Tesla FT-ICR mass spectrometer [34] (NHMFL, Tallahassee, FL) equipped with an external microelectrospray ionization source [35]. Electrospray ionization (ESI) of the samples was performed by use of a NanoMate autosampler/ESI source (Advion Biosciences, Ithaca, NY). The equipment was operated remotely at the University of Birmingham, UK, as described previously [36]. Electrosprayed ions were delivered into the mass spectrometer through a Chait-style atmosphere-to-vacuum interface [37] and externally accumulated [35] in an rf-only octapole. Ions were transferred through multipole ion guides and trapped in an open [38] cylindrical cell (Malmberg-Penning trap) [39]. A front-end resolving quadrupole [40] served to isolate the peptide ion under investigation.

Figure 1. ECD FT-ICR mass spectrum of $[M + 2H]^{2+}$ ions of peptide Lys-Leu₄-Pro-Leu₄. Mass spectrum calibrated against the parent and charge-reduced $[M + H]^+$ ions. A = singly charged ions not removed in isolation; 2ν = second harmonic.

An indirectly heated dispenser cathode (Heat Wave, Watsonville, CA) mounted on the central axis of the system provided the electrons for ECD [41]. A potential of either -2.0 or -5.0 V was applied to the cathode during the irradiation event. A grid situated in front of the filament was kept at -200 V for most of the experiment and pulsed to $+50$ V during the ECD event. The ICR trapping potential was 10 V during the ECD event. The isolated parent ions were irradiated with electrons for 2–20 ms. These parameters correspond to the minimum conditions required for ECD of these peptide ions. For the double resonance experiments, fragment c_n ions were ejected continuously from the ICR cell throughout the ECD and electron cleanup events by resonant single-frequency dipolar excitation at 1019.80 kHz (c_9), 906.70 kHz (c_8), or 793.60 kHz (c_7) (0.05 peak-to-peak voltage (V_{p-p})).

Ions were frequency-sweep (“chirp”) [42] excited and detected in direct mode (512 kword time-domain data). Between 20 and 50 time-domain data sets were

Figure 2. ECD FT-ICR mass spectrum of $[M + 2H]^{2+}$ ions of peptide Arg-Leu₄-Pro-Leu₄. Mass spectrum calibrated against the parent and charge-reduced $[M + H]^+$ ions. A = noise spikes; 2ν = second harmonic.

Table 1. Fragments observed following ECD of $[M + 2H]^{2+}$ ions of KLLLLPLLLL

Fragment ion	m/z_{meas}	m/z_{calc}	$\Delta m/\text{ppm}$
c_3	372.2972	372.2975	0.8
c_4	485.3812	485.3815	0.6
Parent $[M + 2H]^{2+}$	574.9229	574.9229	
c_6	695.5184	695.5184	0.0
c_7	808.6019	808.6024	0.7
c_8	921.6855	921.6865	1.1
c_9	1034.7696	1034.7710	1.3
$[M + H]^+$	1148.8386	1148.8386	

Mass spectrum calibrated against the parent and charge-reduced $[M + H]^+$ ions.

co-added, Hanning apodized, zero-filled once, and subjected to fast Fourier transform followed by magnitude calculation. The experimental event sequence was controlled by a modular ICR data acquisition system (MIDAS) [43]. The FT-ICR mass spectra were internally frequency-to- m/z calibrated [44, 45] with respect to the precursor ion and the charge reduced species (either $[M + H]^+$ or $[M + 2H]^+$) or backbone fragments. The FT-ICR mass spectra were analyzed by use of the MIDAS analysis software package [46].

Results and Discussion

Figures 1–6 show the FT-ICR mass spectra obtained following ECD of $[M + 2H]^{2+}$ ions of the peptides Lys-Leu₄-Pro-Leu₄, Arg-Leu₄-Pro-Leu₄, Leu₄-Pro-Leu₄-Lys, Leu₄-Pro-Leu₄-Arg, Gly₄-Pro-Gly₄-Lys, and Gly₄-Pro-Gly₄-Arg. Summaries of the backbone fragments observed are given in Tables 1 through 6. Figure 7 shows the FT-ICR mass spectrum obtained following ECD of $[M + 2H]^{2+}$ ions of the peptide substance P and its analogs—one containing two lysine residues and the other containing two arginine residues.

The ECD spectra obtained from the peptides Lys-Leu₄-Pro-Leu₄ and Arg-Leu₄-Pro-Leu₄ (Figures 1 and 2; Tables 1 and 2) reveal that the dominant pathway is cleavage to form c fragments. Protonation of peptides occurs at basic amino acid residues (typically lysine and/or arginine, but also in some cases histidine) or on the amino group at the N-terminus. Therefore, the

Table 2. Fragments observed following ECD of $[M + 2H]^{2+}$ ions of RLLLLPLLLL

Fragment ion	m/z_{meas}	m/z_{calc}	$\Delta m/\text{ppm}$
c_3	400.3036	400.3036	0
c_4	513.3874	513.3877	0.6
Parent $[M + 2H]^{2+}$	588.9260	588.9260	
c_6	723.5239	723.5245	0.8
c_7	836.6078	836.6086	0.9
c_8	949.6919	949.6926	0.7
c_9	1062.7740	1062.777	2.2
$[M + H]^+$	1176.8448	1176.8448	

Mass spectrum calibrated against the parent and charge-reduced $[M + H]^+$ ions.

Figure 3. ECD FT-ICR mass spectrum of $[M + 2H]^{2+}$ ions of peptide Leu₄-Pro-Leu₄-Lys. Mass spectrum calibrated against the parent and charge-reduced $[M + H]^+$ ions. 2ν = second harmonic.

peptides Lys-Leu₄-Pro-Leu₄ and Arg-Leu₄-Pro-Leu₄ would be expected to be protonated at the N-terminal amino group and the side chain of the N-terminal amino acid (Lys and Arg, respectively). The charge distribution explains the characteristic feature of the ECD mass spectra, namely, that exclusively N-terminal fragments are observed. Regardless of which proton captures the electron, the charge remains at the N-terminus. Subsequent cleavage of the peptide backbone will result in charged *c* fragments and neutral *z* fragments that can not be detected by the mass spectrometer [47]. No *b* ions were observed for either peptide. The *y*₇ and *y*₉ ions were observed for the Lys peptide. The production of *a*[•] and *y* ions is a minor fragmentation channel in ECD [5].

Figure 3 shows the ECD spectrum obtained from $[M + 2H]^{2+}$ ions of peptide Leu₄-Pro-Leu₄-Lys (See also Table 3). The most abundant fragment peak corresponds to *b*₉ ions. Peaks corresponding to *b*₈ and *b*₇ are observed at a lower abundance. In addition, peaks corresponding to *c*, *z*, and *w* ions are observed. The presence of both N- (*c* ions) and C-terminal fragments (*z* and *w* ions) is indicative of the charging pattern, that is,

Figure 4. ECD FT-ICR mass spectrum of $[M + 2H]^{2+}$ ions of peptide Leu₄-Pro-Leu₄-Arg. Mass spectrum calibrated against the parent and charge-reduced $[M + 2H]^{2+}$ ions. A = noise spikes.

Table 3. Fragments observed following ECD of $[M + 2H]^{2+}$ ions of LLLLPLLLLK

Fragment ion	m/z_{meas}	m/z_{calc}	$\Delta m/\text{ppm}$
<i>z</i> ₄	471.3547	471.3546	0.1
<i>w</i> ₅	540.3761	540.3761	0
Parent $[M + 2H]^{2+}$	574.9229	574.9229	
<i>z</i> ₅ [•]	583.4311	583.4309	0.3
<i>z</i> ₇ [•] – 2(C ₄ H ₈)	681.4410	681.4425	2.2
<i>y</i> ₆	696.5031	696.5024	1.1
<i>z</i> ₇ [•] – C ₄ H ₈	737.5068	737.5051	2.3
<i>w</i> ₇	750.5132	750.5129	0.3
<i>b</i> ₇	776.5647	776.5650	0.3
<i>c</i> ₇	793.5906	793.5915	1.1
<i>w</i> ₈	863.5971	863.5970	0.1
<i>b</i> ₈	889.6500	889.6490	1.1
<i>c</i> ₈	906.6743	906.6756	1.4
<i>y</i> ₈	922.6725	922.6756	3.4
<i>z</i> ₉ [•] – C ₄ H ₈	963.6764	963.6734	3.1
<i>b</i> ₉	1002.7341	1002.7331	0.9
<i>c</i> ₉	1019.7581	1019.7596	1.4
<i>w</i> ₁₀	1089.7672	1089.7651	1.9
$[M + H]^+$	1148.8386	1148.8386	

Mass is spectrum calibrated against the parent and charge-reduced $[M + H]^+$ ions.

protonation at the N-terminal amino group and the side chain of the C-terminal basic residue. The ECD spectrum for the Arg equivalent peptide (Figure 4, Table 4) also reveals the presence of *b* ions, in particular *b*₉; however, these ions do not constitute the major products of ECD. Again, the ECD of doubly protonated

Table 4. Fragments observed following ECD of $[M + 2H]^{2+}$ ions of LLLLPLLLLRR

Fragment ion	m/z_{meas}	m/z_{calc}	$\Delta m/\text{ppm}$
<i>z</i> ₃	386.2758	386.2767	2.3
<i>b</i> ₄	453.3437	453.3441	0.9
<i>w</i> ₄	455.2975	455.2982	1.5
<i>z</i> ₄	498.3525	498.3530	1.0
<i>z</i> ₅ [•] – C ₄ H ₈	555.3739	555.3744	0.9
<i>w</i> ₅	568.3822	568.3823	0.1
$[M + 2H]^{2+}$	588.9260	588.9260	
<i>z</i> ₅ [•]	611.4351	611.4370	3.0
<i>z</i> ₇ [•] – 2(C ₄ H ₈)	709.4503	709.4487	2.2
<i>y</i> ₆	724.5087	724.5085	0.3
<i>z</i> ₇ [•] – C ₄ H ₈	765.5120	765.5113	0.9
<i>b</i> ₇	776.5663	776.5650	1.6
<i>w</i> ₇	778.5197	778.5191	0.7
<i>z</i> ₇ [•]	821.5737	821.5739	0.2
<i>y</i> ₇	837.5934	837.5926	1.0
<i>z</i> ₈ [•] – C ₄ H ₈	878.5980	878.5953	3.0
<i>b</i> ₈	889.6493	889.6490	0.4
<i>w</i> ₈	891.6035	891.6031	0.4
<i>y</i> ₈	950.6754	950.6766	1.3
<i>z</i> ₉ [•] – C ₄ H ₈	991.6816	991.6794	2.2
<i>b</i> ₉	1002.7337	1002.7330	0.7
<i>c</i> ₉	1019.7589	1019.7600	1.0
<i>w</i> ₁₀	1117.7734	1117.7713	1.9
$[M + 2H]^{2+}$	1177.8530	1177.8530	

Mass spectrum is calibrated against the parent and charge-reduced $[M + 2H]^{2+}$ ions.

Figure 5. ECD FT-ICR mass spectrum of $[M + 2H]^{2+}$ ions of peptide Gly₄-Pro-Gly₄-Lys. Mass spectrum calibrated against the parent and charge-reduced $[M + H]^+$ ions. #, denotes diradical.

Leu₄-Pro-Leu₄-Arg ions results in extensive secondary fragmentation and the formation of *w* ions.

The ECD spectra obtained from the peptides Gly₄-Pro-Gly₄-Lys and Gly₄-Pro-Gly₄-Arg are shown in Figures 5 and 6 (see also Tables 5 and 6). The dominant fragmentation channel for the Lys peptide is the production of *b* ions accompanied by the loss of water. In addition, *c*, *z*, and *y* ions are observed. For the Arg peptide, the spectrum is more complex with peaks corresponding to *c*, *z*, *y*, and *b* ions, although the latter do not dominate.

Figure 7 shows the ECD spectra obtained from doubly protonated substance P and its lysine and arginine analogs. The fragments are summarized in Table 7. Each of these spectra reveals dissociation to predominantly *c* fragments with some contribution from the *z*₉ fragments.

The primary purpose of this study was the investigation of the production of *b* ions following electron capture. The *b* ions observed in the ECD spectra presented here were not the product of CID processes. Precursor ion isolation was achieved through use of a

Figure 6. ECD FT-ICR mass spectrum of $[M + 2H]^{2+}$ ions of peptide Gly₄-Pro-Gly₄-Arg. Mass spectrum calibrated against the parent and charge-reduced $[M + H]^+$ ions. #, denotes diradical.

Table 5. Fragments observed following ECD of $[M + 2H]^{2+}$ ions of GGGGPGGGGK

Fragment ion	m/z_{meas}	m/z_{calc}	$\Delta m/\text{ppm}$
z_4	303.1666	303.1668	0.9
c_5	343.1727	343.1730	0.9
Parent $[M + 2H]^{2+}$	350.6725	350.6725	
$b_6 - H_2O$	365.1568	365.1573	1.5
b_6	383.1677	383.1679	0.5
c_6	400.194	400.1945	1.2
$b_7 - H_2O$	422.1785	422.1788	0.7
$z_6^{\#} - C_2H_4$	429.2095	429.2098	0.7
c_7	457.2156	457.2159	0.7
$a_8^{\cdot}$	470.2361	470.2364	0.7
$b_8 - H_2O$	479.1999	479.2002	0.7
c_8	514.237	514.2374	0.7
$b_9 - H_2O$	536.2215	536.2217	0.5
c_9	571.2583	571.2588	0.9
y_8	586.2947	586.2949	0.3
$[M + H]^+$	700.3378	700.3378	

Mass spectrum is calibrated against the parent and charge-reduced $[M + H]^+$ ions. #, denotes diradical.

resolving quadrupole before trapping in the ICR cell. No waveforms (SWIFT, COSE, etc.) were applied; thus, the trapped ions were not excited. Activated ion techniques were not applied. Precursor ions fragmented as a result of low-energy electron irradiation only. To confirm this, for each ECD experiment, a second experiment was performed that was identical except that the

Table 6. Fragments observed following ECD of $[M + 2H]^{2+}$ ions of GGGGPGGGGR

Fragment ion	m/z_{meas}	m/z_{calc}	$\Delta m/\text{ppm}$
$z_3^{\cdot}$	273.1435	273.1437	0.9
y_3	289.1622	289.1624	0.8
z_4	330.1650	330.1652	0.5
c_5	343.1725	343.1730	1.5
y_4	346.1836	346.1839	1.0
$[M + 2H]^{2+}$	364.6756	364.6756	
$b_6 - H_2O$	365.1580	365.1573	1.8
b_6	383.1678	383.1679	0.4
z_5	387.1864	387.1866	0.4
y_5	403.2051	403.2054	0.7
$b_7 - H_2O$	422.1788	422.1788	0.0
c_7 or $z_6^{\#} - C_2H_4$	457.2157	457.2159	0.5
$a_8^{\cdot} + H$ or $z_6^{\#} - CH_2$	471.2315	471.2315	0.2
$b_8 - H_2O$	479.2001	479.2002	0.2
$z_6^{\#} - 2H$	483.2314	483.2316	0.5
$y_6 - 2H$	498.2423	498.2425	0.3
y_6	500.2580	500.2581	0.3
c_8	514.2376	514.2374	0.4
$b_9 - H_2O$	536.2216	536.2217	0.2
$z_7^{\cdot}$	541.2608	541.2609	0.2
y_7	557.2793	557.2796	0.6
c_9	571.2587	571.2588	0.1
z_8	598.2822	598.2823	0.2
y_8	614.3004	614.3010	1.0
$z_9^{\cdot}$	655.3037	655.3034	0.5
$[M + H]^+$	728.3440	728.3440	

Mass spectrum is calibrated against the parent and charge-reduced $[M + H]^+$ ions. #, denotes diradical.

Figure 7. ECD FT-ICR mass spectra of $[M + 2H]^{2+}$ ions of peptides substance P RPKPQQFFGLM-NH₂ (a); KPKPQQFFGLM-NH₂ (b); and RPRPQQFFGLM-NH₂ (c). Mass spectra calibrated against the parent and charge-reduced $[M + 2H]^{++}$ ions. $2v$ = second harmonic.

grid potential remained at -200 V during the notional ECD event, that is, no electrons entered the ICR cell. No fragments were observed for any peptide.

If the production of b ions is not the result of CID processes, these fragments must be the result of electron capture. Uggerud and coworkers recently performed a number of model calculations [25]. Their results suggested that if the precursor ion was N-protonated, the products of electron capture would be b and y ions. It is possible then that the b ions observed in our experiments are the result of fragmentation of N-protonated precursor peptide ions. Alternatively, it may be that the b ions observed are the products of secondary fragmentation, possibly because of infrared irradiation from the heated dispenser cathode, i.e., the b ions result from

cleavage within c ions. To investigate this, we performed double resonance experiments on the peptide Leu₄-Pro-Leu₄-Lys. During the ECD event, and the subsequent electron cleanup event, c_n ($n = 7, 8$, and 9) ions were ejected continuously from the ICR cell by resonant single-frequency dipolar excitation at the frequency corresponding to the particular c_n ion. Any product ion resulting from fragmentation of that c_n ion will not be observed in the resulting mass spectrum. In each case, the putative secondary b_n ion was still present in the ECD mass spectrum. The mass spectrum obtained following simultaneous ECD of $[Leu_4-Pro-Leu_4-Lys + 2H]^{2+}$ and excitation of the c_9 fragment ions is shown in Figure 8. These results suggest that either the b ions are not the result of secondary fragmentation of c ions or that secondary fragmentation of the c ion occurs before ejection.

A common, but minor, fragmentation channel following electron capture by peptide ions results in the production of a ions [5]. Possibly, a ions are the secondary products of unstable b ions and, in the examples presented here, those b ions are stable. With the exception of the Gly-containing peptides, no a ions were observed in the ECD mass spectra. The ECD behavior of the Gly-containing peptides (Figures 5 and 6; Tables 5 and 6) was characterized by extensive secondary fragmentation. The b fragments observed following ECD of the Gly-containing peptides were accompanied by water loss. Water loss from b ions is typically observed for peptides containing an $-OH$ moiety in the amino acid side chain, for example, serine. A possible explanation for the water loss observed in the Gly peptides is that the b ions observed here do not have oxonium or acylium structures, but protonated diketopiperazine structures [48, 49], which do contain an $-OH$ moiety. The extent of b fragmentation, in terms of sequence coverage, was also greater for the Gly-containing peptides. Moreover, the ECD spectra of these peptides revealed c/z -type cleavage within the proline residue (which was not observed for any of the other peptides studied). Those observations may be rationalized by consideration of the lack of side chains in the glycine residues and, hence, secondary structure in the peptide ions.

Another possible explanation for the presence of b ions is as follows: Hydrogen atom loss is a common fragmentation channel following electron capture by peptides. It is conceivable, therefore, that the mechanism for b ion formation involves electron capture by $[M + nH]^{n+}$ with concomitant energy deposition and hydrogen atom loss to yield the excited $[M + (n - 1)H]^{(n-1)+}$ species. Because this species is not a radical, subsequent fragmentation occurs via the "standard" peptide bond cleavage pathway, i.e., the mobile proton pathway [50], seen in traditional MS/MS techniques. The central tenet of the mobile proton model is that fragmentation of most protonated peptides requires the involvement of a proton at the cleavage site, i.e., that the cleavages are "charge directed." As described previ-

Table 7. Fragments observed following ECD of $[M + 2H]^{2+}$ ions of substance P, Lys-substance P, and Arg-substance P

Fragment Ion	Substance P			Lys-substance P			Arg-substance P		
	m/z_{meas}	m/z_{calc}	$\Delta m/\text{ppm}$	m/z_{meas}	m/z_{calc}	$\Delta m/\text{ppm}$	m/z_{meas}	m/z_{calc}	$\Delta m/\text{ppm}$
c_4	496.3354	496.3360	1.1	468.3291	468.3298	1.5	524.3422	524.3421	0.1
c_5	624.3944	624.3946	0.3	596.3878	596.3884	1.0	652.4003	652.4007	
Parent	674.3716			660.3685			688.3747		
c_6	752.4537	752.4531	0.7	724.4477	724.4470	0.9	780.4610	780.4593	2.1
a_7	855.5085	855.5079	0.7						
c_7	899.5227	899.5215	1.3	871.5165	871.5154	1.3	927.5261	927.5277	1.7
c_8	1046.5924	1046.5900	2.2	1018.5890	1018.5838	5.0	1074.5934	1074.5960	2.4
z_9	1078.5716	1078.5630	8.0				1106.5713	1106.5700	1.2
c_9	1103.6119	1103.6110	0.8	1075.6085	1075.6053	3.0	1131.6142	1131.6180	3.3
c_{10}	1216.6965	1216.6960	0.4	1188.6969	1188.6893	6.4	1244.6983	1244.7020	3.0
$[M + 2H]^{+}$	1348.7440			1320.7380			1376.7500		

Mass spectra calibrated against the parent and charge-reduced $[M + 2H]^{+}$ ions.

ously, protonation of peptides typically occurs on a basic amino acid side chain. Energy deposition results in mobilization of the proton to the peptide backbone and dissociation is induced. For peptides Lys-Leu₄-Pro-Leu₄, Arg-Leu₄-Pro-Leu₄, Leu₄-Pro-Leu₄-Lys, Gly₄-Pro-Gly₄-Lys, and Gly₄-Pro-Gly₄-Arg, the peak corresponding to the $[M + H]^+$ ions was significantly more intense than that corresponding to $[M + 2H]^{+}$ ions, i.e., H[•] loss was prevalent. For peptide Leu₄-Pro-Leu₄-Arg, the peaks corresponding to $[M + H]^+$ and $[M + 2H]^{+}$ have approximately equivalent intensities.

Interestingly, the most abundant b ions were those adjacent to the basic residues, i.e., b_9 for those peptides studied here and b_8 for the peptides in our previous study [28]. Closer inspection of the peptide studied by Chan and coworkers [32], Lys-Gly₃-Trp-Gly₃-Lys, reveals that the b_8 ion is observed following electron capture, i.e., the peptide bond adjacent to the C-terminal Lys residue was cleaved. Moreover, the b ions observed in the ECD of $[M + 4H]^{4+}$ ions of Ac-(Ala-Lys-Ala-Ala-Lys)₃-Ala-NH₂ studied by Iavorone [33] all result from cleavage of the peptide bonds adjacent to Lys residues. Electron capture is most likely to occur at

the least basic charged site [51]. For the peptides Leu₄-Pro-Leu₄-Lys, Leu₄-Pro-Leu₄-Arg, Gly₄-Pro-Gly₄-Lys, and Gly₄-Pro-Gly₄-Arg, electron capture is predicted to occur at the N-terminus. Thus, the b ion formation observed appears to reflect the position of the proton in the charge-reduced species. The b ions were not observed for peptides where the basic residue was at the N-terminus. That observation is unsurprising in light of the foregoing explanation; either additional H atom loss would be observed or a b_1 ion would result. The cyclic structure of b ions negates the formation of b_1 ions and these species are never observed.

The presence of b ions was more pronounced for Lys-containing peptides than for Arg-containing peptides. There are two points to consider. First, the recombination energies for *N*-acetyl-lysine amide and *N*-acetyl-arginine amide cations are 2.84 and 2.44 eV, respectively [52]. The total recombination energies depend on the conformation of the peptide ions. Valentine et al. [53] calculated that peptide ions of the form $[(Xxx)_n\text{-Lys} + H]^+$ have very similar cross sections to the analogous $[(Xxx)_n\text{-Arg} + H]^+$ ions. If the same is true of doubly charged species, and then the recombination energy would be greater for the Lys-containing peptides than the Arg-containing peptides. Thus, greater b ion production would be expected for the Lys-containing peptides. Second, if the peptides are fragmenting according to the proposed mechanism, that is, H[•] loss followed by peptide bond cleavage according to the mobile proton model, then the energy required for mobilization of a proton from arginine is greater than that required for mobilization from lysine. Greater fragmentation would be predicted for Lys-containing peptides.

The ECD spectra of doubly protonated substance P and its analogs did not reveal any b fragmentation. Unlike the other peptides studied, there are three possible charge patterns in $[M + 2H]^{2+}$ ions of substance P: (1) protonation at the N-terminus and Arg side chain, (2) protonation at the N-terminus and the Lys side chain, and (3) protonation of the Arg and Lys side chains. The possible charge sites are all close to the N-terminus; nevertheless, the b_2

Figure 8. Selected region of ECD mass spectrum of $[M + 2H]^{2+}$ ions of Leu₄-Pro-Leu₄-Lys (m/z 990–1060). The c_9 fragment ion was continually ejected throughout the ECD and subsequent electron cleanup event. The b_9 ion is observed in the mass spectrum.

ion might be expected if electron capture occurred at the protonated lysine (position 3). It may be that the low mass fragments are not being detected as the expected c_2 ions are also not observed in these spectra. The most stable conformer of doubly protonated substance P has an internally solvated lysine side chain [54]. According to the proposed mechanism, for b fragments to be produced, hydrogen atom loss, rather than transfer, must occur. For internally solvated amide carbonyl groups, however, hydrogen transfer is favored. Although peaks corresponding to $[M + H]^+$ ions were observed for these three peptides, the $[M + 2H]^+$ peaks predominated. Additionally, capture of an electron at the Lys residue provides recombination energy 3.7 eV, whereas capture at the Arg residue provides recombination energy 4.23 eV [52].

The results suggest that the presence of b ions in ECD spectra result from a balance of several factors. Clearly, if electron capture occurs at or adjacent to the N-terminus, b ions will not be observed. For the model peptides (Xxx_n-Lys) and (Xxx_n-Arg), whose cross sections are similar, the prevailing factors for b ion production appears to be recombination energy and proton affinity. Internal solvation also appears to play a crucial role. Hydrogen atom transfer will be favored over hydrogen atom loss for internally solvated amide carbonyl groups. Syrstad and Tureček's calculations suggest that the charged guanidinium group in protonated arginine is much less effectively internally solvated than the ammonium group in protonated lysine [27]. On the other hand, more energy is required to mobilize an Arg-associated proton than a Lys-associated proton.

Conclusion

In summary, we have shown that b fragment ions may result from electron capture by peptide ions. The proposed mechanism involves electron capture and energy deposition, followed by hydrogen atom loss and subsequent dissociation of the nonradical species by cleavage of the peptide backbone. Our results suggest that b ions in ECD mass spectra are not the product of secondary dissociation of c ions. Detection of the presence of b ions in ECD spectra may be used in determining the charged site(s) in charge-reduced species. That hypothesis is corroborated by the findings of Chan and coworkers [32] and Iavarone et al. [33]. Both peptide ion conformations, recombination energy, and proton affinity appear to play roles in the electron capture-mediated production of b ions.

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