Secondary fragmentations of three synthetic peptides (human αA crystallin peptide 1-11, the deamidated form of human βB2 crystallin peptide 4-14, and amyloid β peptide 25-35) were studied in both electron capture dissociation (ECD) and electron-transfer dissociation (ETD) mode. In ECD, in addition to c and z- ion formations, charge remote fragmentations (CRF) of z- ions were abundant, resulting in internal fragment formation or partial/entire side-chain losses from amino acids, sometimes several residues away from the backbone cleavage site, and to some extent multiple side-chain losses. The internal fragments were observed in peptides with basic residues located in the middle of the sequences, which was different from most tryptic peptides with basic residues located at the C-terminus. These secondary cleavages were initiated by hydrogen abstraction at the α-, β-, or γ-position of the amino acid side chain. In comparison, ETD generates fewer CRF fragments than ECD. This secondary cleavage study will facilitate ECD/ETD spectra interpretation, and help de novo sequencing and database searching. (J Am Soc Mass Spectrom 2010, 21, 646–656) © 2010 Published by Elsevier Inc. on behalf of American Society for Mass Spectrometry

Radical induced gas-phase fragmentation processes in peptides/proteins have been extensively studied. Many methods have been used to generate free radicals in peptides/proteins, including homolytic cleavages of covalent bonds that couple the chemical/chromophore groups or radical initiators to the peptides, dissociation of the noncovalent complexes of peptides, and peptide ion-electron/ion-ion interaction methods. The weak covalent bonds in the lysine peroxycarbamates (LPC) and the free radical initiator Vazo 68-peptide conjugates may be collisionally activated to generate free radicals in peptide ions [1–3]. Collision induced dissociation (CID) was also used to generate radical cations from the S- and N-nitrosopeptides or sodiated nitrate esters of serine and homoserine derivatives [4, 5]. Site-specific radical directed dissociations can be induced by photodissociation (PD) of a carbon-sulfur (C=S) bond introduced at phosphorylated serine and threonine residues through elimination of the phosphate in basic solution followed by addition of a thiol with an attached naphthyl based chromophore group [6], or PD/CID of a carbon-iodine (C–I) bond at iodinated tyrosine, and to a lesser extent, iodinated histidine [7]. CID of the ternary complex [Metal(L)(M)]

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common fragmentation channel is the α-cleavage of the Cα-Cα bond of the N-terminal residue of the z· ion, resulting in the formation of w· ions. However, the radical may also migrate to other positions via hydrogen abstraction to induce bond cleavages remote from the initial radical site, similar to the dissociation pathways observed in the vacuum ultraviolet (VUV) photodissociation of peptide ions, and the surface induced dissociation (SID) and CID of peptide radical cations [22, 23]. In ECD, the likelihood of radical rearrangement generally decreases as the distance between the primary cleavage site and the hydrogen abstraction site along the peptide backbone increases, although through-space hydrogen abstraction does occur [24, 25]. Neutral losses from the reduced precursor ions and secondary side-chain losses from z· ions in ECD have been studied extensively in linear peptides [26–33]. Loss of the entire or partial side chain from z· ions was considered to be associated with the γ or α-radical formation on the corresponding amino acid, respectively [28, 29]. Because these secondary fragmentations do not involve the charge directly, and the bond cleavage sites are spatially remote from the charge carrier site, they can be considered as “charge-remote fragmentations” (CRFs) [11, 34–39]. CRFs are not as common in ETD, presumably because of the less energy deposited upon electron transfer, as well as the collisional cooling effect present in the ion trap commonly used to carry out ETD experiments. However, when z· ions from ETD were collisionally activated, extensive radical induced fragmentations were also readily observed [40]. CRF may also lead to secondary backbone cleavages, as evident in the ECD of doubly charged cyclic peptides, where multiple backbone cleavages and side-chain losses were observed with the capture of a single electron [41]. A free radical cascade (FRC) mechanism was proposed, in which the initial α-radical can propagate along the peptide backbone to generate another α-radical with a stable even-electron neutral as the leaving group. FRC also predicts the formation of internal fragments in ECD of linear peptides. However, since most peptide ions studied in ECD experiments are electrospray ionization (ESI)-generated tryptic peptides with charges located near the termini, the internal fragments, even if formed, would be neutral and undetectable. Nevertheless, for non-tryptic peptides, or for tryptic peptides with basic residue(s) located in the middle of the sequence either due to missed cleavage(s) or the presence of histidine residue(s), internal fragments may be detected. To test this hypothesis, three synthetic peptides were investigated by ECD and ETD. The internal fragment ions were observed and the postulated mechanisms were discussed. Side-chain losses were also investigated to help further understand the radical’s fate in z· ions.

**Results and Discussion**

ECD measurements of three synthetic peptides were performed, with one mimicking a tryptic peptide and two being non-tryptic peptides. Figure 1 shows the ECD spectrum of the tryptic peptide (αA crystallin peptide, 1-11) with a histidine residue in the middle of the sequence. Figure 2 and Figure 3 show the ECD spectra of two non-tryptic peptides (amyloid β peptide, 25-35 and the deamidated form of βB2 crystallin peptide, 4-14) each containing a lysine residue in the middle of the peptide sequence. In the ECD and ETD spectra of this study, peaks corresponding to both the charge-reduced molecular ions and its hydrogen loss products ([M + nH]n–1+) and [M + (n–1)H]n–1+) were observed. These spectra were dominated by c and z· ion series, as expected. There were also several b ions present in the spectra, particularly in Figure 2, in agreement with previous studies [45, 46]. In addition, peaks corresponding to entire and partial side-chain losses from z· ions, as well as internal fragments were observed. In comparison, fewer CRF fragments were observed in the ETD spectra of the deamidated form of
βB2 crystallin peptide, 4-14 (Figure 4) than in its ECD spectrum (Figure 3). Complete peak lists and assignments are available in Supplementary Tables 1–5, which can be found in the electronic version of this article. ETD spectra of the doubly charged αA crystallin peptide and amyloid β peptide are shown in Supplementary Figures S1 and S2, respectively.

Figure 1. ECD spectrum of αA crystallin peptide (1-11). * Represents the electronic noise peak or salt adducts, ω represents the harmonic peaks. Peaks marked with “-amino acid residue” represent the entire side-chain losses, and the partial side-chain losses are represented by the molecular formulas of the departing group(s). Side-chain loss means loss from the charge-reduced molecular ions. The cleavage pattern is shown as the inset.

Entire Side-Chain Loss from z· Ions Associated with γ-Radical Formation on the Side Chain

For amino acids containing γ-hydrogen(s) on their side chains, entire side-chain losses from z· ions may be observed, usually at sites remote from the primary backbone cleavage sites [11, 12, 23, 28]. These amino

Figure 2. ECD spectrum of amyloid β peptide (25-35). Peak labeling follows the same convention as in Figure 1. The cleavage pattern is shown as the inset. (Reprinted with permission from reference [47].)
acids include: Ile/Leu, Lys, Arg, Glu, Gln, Cys, Met, and Val. Scheme 1 illustrates a possible mechanism for the loss of the entire side chain, using glutamine as an example. After the initial z· fragment formation, the α-radical on the N-terminal carbon of the z· fragment may abstract one hydrogen from the γ-carbon of the glutamine side chain, and the newly formed γ-radical can undergo an α-cleavage reaction and release the side chain as an even-electron, neutral species from the peptide, leaving the radical on the peptide backbone. For example, in the ECD spectrum of the αA crystallin peptide (1-11), the entire glutamine side chain was lost from the z· and z· ions, and the lysine side chain was lost from the z· ions (Figure 1); in none of these cases is the backbone cleavage site adjacent to the site of side-chain loss. In other ECD spectra, glutamic acid side-chain losses from z· fragments were also detected with low abundance (data not shown). In the ECD spectrum of amyloid β peptide (25-35), peaks corresponding to entire side-chain losses from z· fragments of leucine/isoleucine, or methionine were also observed (Figure 2) [47].

In the ECD spectrum of the αA crystallin peptide (1-11) (Figure 1), a series of peaks corresponding to loss of the entire side chain of a tryptophan residue (129 Da) from the z· fragments were observed, some of which have relatively high abundance, such as the z·-Trp at m/z 911. For all of these z·-Trp ions, the backbone cleavage sites were remote from the tryptophan residue. This remote loss of tryptophan side chain from z· ions has been reported previously in ECD and ETD studies, the latter requiring additional collisional activation [28, 40]. Siu and coworkers have observed the 129 Da loss of the tryptophan side chain in the CID mass spectra of the M+ ion of WGG formed by the collisional induced dissociation of the [CuII(dien)M]2+ complex [9], where they proposed that the tryptophan side chain was lost as a carbene species, following the 1,4-proton transfer that puts the charge on the backbone carbonyl and the radical on the β-carbon. Although there is no γ-hydrogen in the common canonical representation of the tryptophan structure as previously suggested [23, 28], a tautomeric form of tryptophan does contain a γ-hydrogen, which can be used to explain the abundant tryptophan side-chain losses present here, as illustrated in Scheme 2. This postulated mechanism may also account for the side-chain loss from tyrosine residue (106 Da), as seen in both ECD of protonated peptide ions [28] and CID of peptide radical ions [8]. Phenylalanine does not have a hydroxyl group to generate the tautomeric structure like tyrosine, and loss of the entire phenylalanine side chain from z· ions has seldom been observed, except once as reported, but under unusual conditions [48]. The histidine residue can assume a γ-hydrogen containing tautomeric form as well, which may lead to a complete side-chain loss of ~80 Da, although it has not been observed in ECD to date. Nonetheless, histidine side-chain loss has been reported in the dissociation of the hydrogen deficient peptide radicals [12], as well as in the CID and SID spectra of M+ ions [11]. This potential ~80 Da loss is different from the ~82 Da loss from the charge reduced species reported in previous studies [26, 32]. The ~82 Da loss proceeds via a different mechanism, where the charge neutralization at the protonated imine nitrogen site generates a stable carbon radical to induce loss of the entire side chain. It is important to note that these neutral side-chain losses are different from the cationic
Side-chain losses in the EID spectra of protonated aromatic amino acid or peptides containing aromatic moieties, where the electron ejected by EI is typically from the aromatic ring itself, leaving both the charge and the radical on the aromatic ring. This radical cation may rearrange to lose a cationic species, which is 1 Da higher than that of the neutral loss (tryptophan: 130 Da, phenylalanine: 91 Da, tyrosine: 107 Da, and histidine: 81 Da) [14].

Multiple side-chain losses from \( z \)-ions were also observed in ECD spectra. For example, in the ECD spectrum of the amyloid \( \beta \) peptide (25-35), peaks corresponding to the losses of Ile/Leu and Met side chains from the \( z_8 \) fragment were observed (\( z_8 \) - Ile/Leu-Met) (Figure 2). This is expected, as loss of the entire side chain leaves the radical on the backbone, which may induce further fragmentations. As long as the radical still remains on the backbone, the side-chain loss cascade may continue.

**Figure 4.** ETD spectra of the deamidated form of \( \beta \)B2 crystallin peptide (4-14) generated from 2+ charge state (a) and 3+ charge state (b). * Represents two fluoranthene molecules. Side-chain loss means loss from the charge-reduced molecular ions. Cleavage patterns are shown as the insets.
Partial Side-Chain Loss from \( z \)-Ions Associated with \( \alpha \)-Radical Formation on the Side Chain

Furthermore, in Figure 1, there were a series of peaks corresponding to the partial side-chain losses of glutamine residue (C\(_2\)H\(_4\)NO) from \( z \)-ions, with the glutamine residue located either directly at the backbone cleavage site or several amino acids away from it. In this peptide, the \( z_6^\alpha \)-C\(_2\)H\(_4\)NO fragment was assigned as the w\(_6 \) ion resulting from the direct cleavage of the C\(_\beta\)-C\(_\gamma\) bond adjacent to the initial radical site, because the side-chain loss occurred at only glutamine residue in the sequence. The other partial side-chain losses of glutamine from \( z \)-ions had to be formed via through-space radical rearrangement. The detailed mechanisms for the partial glutamine side-chain losses from \( z \)-ions are shown in Scheme 3, similar to those proposed by other groups [12, 23, 28, 41]. The \( \alpha \)-carbon radical, either in the original position or as a result of the \( \alpha \)-hydrogen abstraction will initiate an \( \alpha \)-cleavage reaction to break the bond between the \( \beta \)- and \( \gamma \)-carbons of the side chain. This will induce the partial loss of the side chain while the radical remains on the lost neutral species, and an even-electron species is formed for the remaining \( z \) fragment. Thus, the \( \alpha \)-radical, N-terminal to the \( z \)-fragment may induce the partial side-chain losses in ECD via through-bond (w ion formation, Scheme 3a) or through-space (Scheme 3b) mechanisms. The remaining \( z \) fragments contain no radicals to initiate further reaction.

A series of partial glutamic acid side-chain losses (C\(_2\)H\(_3\)O\(_2\)) from the corresponding \( z \)-ions were observed in the ECD spectrum of the deamidated form of the \( \beta \)B2 crystallin peptide (4-14) as well (Figure 3). For this peptide, some of the side-chain loss fragment ions contain multiple glutamic acid residues located either directly at, or remote from, the backbone cleavage sites. The \( z_8^\alpha \)-C\(_2\)H\(_3\)O\(_2\) and \( z_{10}^\alpha \)-C\(_2\)H\(_3\)O\(_2\) ions may be generated either as the w \( \) ions or formed following radical migration, while the other \( z \)-C\(_2\)H\(_3\)O\(_2\) ions had to be formed after through-space hydrogen abstraction. The high-frequency of the partial side-chain losses of \( z \)-ions indicates that, after the formation of \( z \)-ions in ECD, the radical on the \( z \)-ions will have ample time to migrate and induce further cleavages, without the need of additional activation. These secondary fragmentations usually do not depend on the charge location in the peptides. Thus, the CRF may be a common pathway for partial side-chain losses from \( z \)-ions, especially in

![Scheme 1](image1.png)  
Scheme 1. Entire side-chain loss from \( z \)-ions via gamma hydrogen abstraction.

![Scheme 2](image2.png)  
Scheme 2. Entire side-chain loss from \( z \)-ions via \( \gamma \) hydrogen abstraction involving aromatic amino acids.
tryptic peptides, which usually have a charge carrier at the C-terminus that permits the detection of secondary C-terminal fragments.

This phenomenon has important implications in de novo sequencing. A specific side-chain loss from z· ions does not warrant the assignment of that amino acid at the cleavage site because the loss may occur at remote sites. For example, in the differentiation of Leu/Ile, it is not sufficient to identify Leu/Ile solely based on the mass difference between the w- and the corresponding z· ions because the partial side-chain loss of Leu/Ile may not come from the N-terminus of the z· ions at all [27, 49]. In the ECD spectrum of the amyloid β peptide (25-35) (Figure 2), partial side-chain losses of leucine and isoleucine (C 3H7 and C 2H5) from z 9· fragment were observed to occur several residues away from the backbone cleavage site. In addition, for the partial side-chain losses of the leucine residue from z· ions in the ECD spectrum of the deamidated form of βB2 crystallin peptide (4-14) (Figure 2), the backbone cleavage sites were seven or eight residues away from the C-terminal leucine. Thus, it is important to assign the residue as a Xle (meaning either Leu/Ile) based on the mass difference between adjacent backbone fragment ions before looking at the side-chain loss for differentiation of Leu and Ile residues, and if several different Leu/Ile residues exist on the same peptide, the assignments may be ambiguous. In addition to Ile/Leu isomer differentiation, ECD can also be used to distinguish aspartic acid (Asp) and isoaspartic acid (isoAsp) based primarily on the presence of diagnostic c + 57 and z· − 57 ions for isoAsp containing peptides [50–54].

The Asp/isoAsp result is likely to be less affected by secondary side-chain cleavages as it results from the primary backbone cleavage of the Cα–Cβ bond, but for cases where several Asp/isoAsp residues are available in the same peptide, similar ambiguity to the Leu/Ile question discussed above could arise.

It has been suggested that α-hydrogen abstraction proceeds via a stepwise mechanism [29]. However, in the current study, partial side-chain loss of methionine residue (C 2H5S) from the z 8· ions was observed abundantly (Figure 2) with the methionine residue located at the C-terminus of the amyloid β peptide (25-35), which was seven residues away from the backbone cleavage site. This suggests that α-hydrogen abstraction may also proceed through space in a single step and that the abundance of the CRF fragments is influenced by both the spatial proximity and the sequence proximity of the amino acid side chain to the backbone cleavage site.

Similar through-space hydrogen transfer may also occur in γ-hydrogen abstraction leading to the entire side-chain losses from residues distant from the original radical site. This hypothesis is supported by the presence of a strong peak corresponding to the z 8·-Trp ion (Figure 1), which is not only much more abundant than the smaller z n·-Trp (n = 5–7) ions despite the comparable intensity of the related z· ions, but also has an intensity higher than that of any other side-chain losses from z 8· ions, even though the threonine and the glutamine residues are both closer to the backbone cleavage site than the tryptophan residue. Of course, one has to take into account the reactivity of the originally formed α radical and the stability of the

![Scheme 3. Partial side-chain loss from z· ions via α hydrogen abstraction [through-bond, (a) and through-space, (b)].](image-url)
radical in its new position, which can also affect the abundance of the side-chain losses. Nevertheless, the abundant distant side-chain losses observed in the present study underscore the importance of gas-phase peptide ion conformations in secondary fragment ion formations in ECD.

In addition, it was found that the w-ions are not always more abundant than the same side-chain losses in longer fragment ions. For example, in Figure 1, the most abundant partial Gln side-chain loss was from the z_8^- ion, rather than the z_6^- ion, the latter of which produced w_6. This is in contrast with the observation in a previous study [28], where the intensity of w_5 ions was found to be consistently at least twice as high as those resulting from the same side-chain loss from larger z_n^- ions, where n ≥ 6. In the previous study, ECD was applied to synthetic peptides with mostly glycine residues, which can only induce limited side-chain interactions. In more complicated peptides as those studied here, gas-phase conformations will influence the secondary fragment ion abundances, even to the extent that could invalidate the criterion for differentiating w ions from u ions as proposed in earlier studies [28, 55].

**Internal Fragments Associated with β-Radical Formation on the Side Chain from z^- Ions**

Four peaks corresponding to internal fragments were found in the ECD spectrum of the deamidated βB2 crystallin peptide (4-14) (Figure 3). Three of them, z_8^-c_8/z_7^-c_9, z_8^-c_9, z_10^-c_8/z_9^-c_9 are even-electron species, and one (z_8^-a_9) contains a radical. The internal fragments discussed in this study were labeled by the ion types at both ends, with each named after the backbone fragment ion that would have been generated, had the other end been the uncleaved C- or N-terminus of the peptide. The N-terminal ends of all these internal fragments result from N-C_ο bond cleavage, while the C-terminal ends mimic either a c^- or an a^-type fragment ion. This suggests that the internal fragment was generated from a z^- ion with its radical initiating the secondary cleavage on the peptide backbone. Scheme 4 illustrates the postulated mechanisms for the internal fragment formation. The z^- radical can abstract a hydrogen atom from the β-carbon of the amino acid side chain to generate a β-radical, which can then induce α-cleavage to generate the secondary cleavage on the peptide backbone. If the α-cleavage occurs toward the C-terminal side of the β-radical, it will generate a z-a^- type internal fragment ion (Scheme 4a). However, the resulting internal fragment would be an even-electron species, which is inconsistent with the experimental observation. The α-cleavage may also proceed toward the N-terminal side of the β-radical, which will generate a z-c^- type internal ion (Scheme 4b). After the secondary backbone cleavage, the radical on the NH- group is not stable, and it can abstract a hydrogen via the intra- or inter-molecular hydrogen transfer to become an even-electron species (pathway I in Scheme 4b). According to the literature, histidine and glutamine residues in c ions are good H^- donors for H^- transfer to z^- ions [56]. In this deamidated βB2 crystallin peptide (4-14), the histidine residue is located at the N-terminus, which could be an

![Scheme 4](image-url)
active H· donor. The unstable N-radical may also lose an O=C=NH molecule to generate a radical z·a· type internal fragment, such as the z8·a9· ion observed here (pathway II in Scheme 4b).

In the ECD spectrum of the αA crystallin peptide (1-11) (Figure 1), one internal fragment z8·c9· was also observed with double H· losses, which is promoted when a threonine residue is located C-terminal to the backbone cleavage site [56]. In addition, there were two internal fragments, z10·a9· and z10·b10·, in the ECD spectrum of amyloid β peptide (25-35) (Figure 2). The internal fragment z10·a9· also had double H· loss because of a serine residue located C-terminal to the backbone cleavage site. Radical induced b/y cleavage has also been discussed in the literature, where a mechanism involving the McLafferty rearrangement was proposed to explain the unusual radical y ions observed in the CID spectra of ETD generated z· ions [40]. Similar to explain the unusual radical y ions observed in the 

Figure 1 shows the ETD spectrum of the doubly charged deamidated βB2 crystallin peptide (4-14) were performed, and another internal fragment z8·c9· had very low abundance and thus is not labeled. Although the internal fragment z8·c9 and the z8· ion produced by ECD can be easily distinguished based on their accurate masses measured by the FT-ICR mass analyzer, such differentiation is impossible in ETD performed in a low-resolution ion trap (Figure 4b). It is also unclear whether the internal fragments in the ETD of the triply charged αA crystallin peptide (1-11) and amyloid β peptide (25-35) were also performed, and they both showed significantly reduced secondary fragmentations (Supplementary Figures 1 and 2, respectively). This reduced CRF fragmentation in ETD could be beneficial, as it will make database searching and/or de novo sequencing a less complex task. On the other hand, ECD’s ability to generate w ions and other secondary fragments makes it a valuable tool for isomer differentiation, which may otherwise be difficult when using ETD alone.

Conclusion

Two non-tryptic peptides and one tryptic peptide with histidine in the middle were used as model peptides to study the secondary fragmentation in ECD/ETD. Radical rearrangement of z· ions may proceed through three possible pathways: α-radical formation along the backbone can induce partial side-chain losses from z· ions; β-radical formation in the side chain may produce internal fragments; γ-radical formation in the side chain will generate entire side-chain losses from z· ions, and this may induce further radical rearrangement, leading to multiple side-chain losses. All three secondary fragmentations took place at sites remote from the charge sites. Fewer CRF fragments were observed in ETD than in ECD. Entire and/or partial side-chain losses from z· ions in ECD can provide additional information for isomer differentiation, help with de novo sequencing, and increase the confidence of database searching. Although in general they cannot be used to identify the location of certain amino acids, they do indicate the existence of specific amino acids. The current study also indicates that the low occurrence of internal fragments in peptide ECD/ETD spectra is likely due to several
reasons: lack of charge carriers in the middle of the sequence of the commonly studied tryptic peptides; the relatively higher Cα-H BDE than the Cα-H BDE, as a β-radical is needed to initiate the secondary backbone cleavage; and the low stability of the radical intermediates involved. Understanding the secondary fragmentation of z· ions is important for the interpretation of ECD spectra with high confidence and accuracy because in some peptide ECD spectra, many major peaks are generated from entire/partial side-chain losses of z· ions. The existence of these CRFs may aid the peptide identification, but could also complicate database searching.

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Appendix A Supplementary Material

Supplementary material associated with this article may be found in the online version at doi:10.1016/j.jasms.2010.01.001.

References


