

## RESEARCH ARTICLE

# Electron Transfer Dissociation (ETD) of Peptides Containing Intrachain Disulfide Bonds

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## Abstract

The fragmentation chemistry of peptides containing intrachain disulfide bonds was investigated under electron transfer dissociation (ETD) conditions. Fragments within the cyclic region of the peptide backbone due to intrachain disulfide bond formation were observed, including: c (odd electron), z (even electron), c-33 Da, z+33 Da, c+32 Da, and z-32 Da types of ions. The presence of these ions indicated cleavages both at the disulfide bond and the N-C $\alpha$  backbone from a single electron transfer event. Mechanistic studies supported a mechanism whereby the N-C $\alpha$  bond was cleaved first, and radical-driven reactions caused cleavage at either an S-S bond or an S-C bond within cysteinyl residues. Direct ETD at the disulfide linkage was also observed, correlating with signature loss of 33 Da (SH) from the charge-reduced peptide ions. Initial ETD cleavage at the disulfide bond was found to be promoted amongst peptides ions of lower charge states, while backbone fragmentation was more abundant for higher charge states. The capability of inducing both backbone and disulfide bond cleavages from ETD could be particularly useful for sequencing peptides containing intact intrachain disulfide bonds. ETD of the 13 peptides studied herein all showed substantial sequence coverage, accounting for 75%–100% of possible backbone fragmentation.

**Key words:** Disulfide bond, Electron transfer dissociation, Post-translational modification, Peptide sequencing, Mass spectrometry

## Introduction

Disulfide bond formation is a frequently occurring post-translational modification (PTM), critical to protein folding and structural stability [1]. Characterization of proteins containing disulfide bonds remains challenging for tandem mass spectrometry based on collision-induced dissociation (CID). The difficulty is caused by the fact that disulfide bonds rarely dissociate in the presence of mobile protons [2]. Therefore, two amide bonds must be cleaved to generate one sequence fragment. Due to the random nature of cleaving two amide bonds, CID spectra of disulfide-linked peptides are typically difficult to interpret and provide limited information for the backbone region within the disulfide loop. By far, the most widely-used approach for characterizing disulfide-containing proteins involves multi-

enzyme digestion and disulfide bond reduction/alkylation prior to mass spectrometric analysis [3]. With the disulfide bond already cleaved, rich sequence information can often be obtained from CID of disulfide-reduced peptides. Sophisticated strategies, including partial disulfide bond reduction or chemical modification combined with tandem mass spectrometry, are needed in order to elucidate disulfide connecting patterns [4]. Generally, the whole process of identification and characterization of proteins containing disulfide bonds is time and sample consuming.

Forgoing solution reduction allows the opportunity of characterizing the connecting patterns of disulfide bonds. Many efforts have been directed toward developing gas-phase dissociation methods to characterize intact disulfide bonds within peptides or proteins. Preferential cleavage of disulfide bonds over peptide backbone bonds under CID conditions has been reported for deprotonated peptides [5, 6] and metal cationized peptides [2, 7–9]. Unique fragments

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associated with disulfide bond cleavages have been used to identify the presence of disulfide bonds within unknown proteins [9, 10]. Disulfide bonds can be selectively excited and cleaved by photons at 157 nm [11] and at 266 nm [12]. When collisional activation is applied after photon dissociation, backbone fragmentation takes place, providing rich sequence information. Electron-based dissociation techniques, such as electron capture dissociation (ECD) [13, 14] and electron detachment dissociation (EDD) [15], have shown preferential cleavage of disulfide bonds over backbone (N–C $\alpha$ ) fragmentation. Electron transfer dissociation (ETD) is an analogue of ECD, conducted on electron dynamic ion trap instruments within the frame of ion/ion reactions [16]. The dissociation chemistry of ETD is similar to ECD in many aspects, including competitive dissociation at disulfide bonds within peptide or protein ions [17, 18]. Recent reports have demonstrated that rich structural information can be obtained by using a multiple fragmentation approach involving ETD and CID, allowing for determination of complicated disulfide linkage patterns within recombinant therapeutic proteins [19, 20].

Given the increasing use of ECD and ETD for studying peptides or proteins containing disulfide bonds, it is important to understand the underlying principles. Several mechanisms have been put forward to account for disulfide bond cleavage in ECD or ETD. The “Coulomb assisted dissociation model” suggests the possibility of direct electron attachment at a disulfide bond, assisted by positive charges in close proximity [21, 22]. Alternatively, an electron can be first attached to a charged site and then transferred to the disulfide bond, either through-space or through-bond, to induce cleavage [23]. For these mechanisms, a subsequent proton transfer is needed to form a thiyl radical (–S•) and a sulfhydryl (–SH) group at the cleavage site. Peptides containing interchain disulfide bonds have been more frequently studied due to the ease of detecting disulfide bond cleavage. So far, very limited experimental data have been collected on ECD or ETD of peptides containing intrachain disulfide bonds [24, 25].

Peptides with intrachain disulfide bonds are often encountered in biological systems, functioning as toxins [26], hormones [27], and defensins [28]. The existence of intrachain disulfide bonds cyclizes the peptide backbone, introducing a different scenario to ECD or ETD as compared to peptides having interchain disulfide bonds. Recently, Mentinova et al. showed that consecutive disulfide bond cleavage and N–C $\alpha$  cleavage could be induced within a single ETD event for an intrachain disulfide-linked peptide (somatostatin), but the order of the two cleavages was not suggested [24]. In this study, we systematically investigated ETD of a series of peptides having intrachain disulfide bonds. The purpose of these experiments was to draw a general picture of the dissociation phenomena and obtain a better understanding of the dissociation chemistry, including the formation and ultimate fate of the radical site. A total of 13 intrachain disulfide linked peptides were studied by ETD

(sequences listed in Table 1). These peptides are biologically active due to intrachain disulfide bond formation, and most of them have their backbone fully enclosed by the intrachain disulfide bond. Note that this type of peptide poses challenges for mass spectrometric analysis, since no sequence ions can typically be obtained from conventional CID experiments. Therefore, understanding the ETD fragmentation chemistry of these intact peptides is critical in maximizing the utility of ETD in the characterization of disulfide-linked peptides, as well as shedding light on the top-down or middle down-approach to proteins containing disulfide bonds.

## Experimental

Peptide and protein samples were purchased from AnaSpec (San Jose, CA, USA) and Sigma-Aldrich (St. Louis, MO, USA) and used without purification. Table 1 lists each peptide with its name and single letter sequence. Working solutions of each peptide were prepared to a final concentration of 10  $\mu$ M in 50/49/1 (vol/vol/vol) methanol/water/acetic acid solution for positive mode nanoelectrospray ionization (nanoESI), using a home-built source. All mass spectra were collected on a Velos LTQ mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) equipped with ETD capability. The lens conditions of the instrument were optimized for peptide ion and ETD reagent signals. ETD reaction time was around 100 ms. Subsequent CID was performed on selected product ions. Supplementary activation during ETD experiments was also employed to dissociate ~90 % of the first generation odd-electron charge reduced peptide ions, with values ranging from 15–25

**Table 1.** List of Peptides Containing Intrachain Disulfide Bonds

label	sequence	name
1	C T T H W G F T L C	CTT, gelatinase inhibitor
2	C L P T R H M A C	CD154 blocking peptide
3	K T K C K F L K K C	Endotoxin inhibitor
4	C G R R A G G S C	IL-11R $\alpha$ binding peptide
5	C N G R C-NH <sub>2</sub>	NGR, peptide 3
6	C G N K R T R G C	LyP-1, peptide 1
7	C Y A A P L K P A K S C	IGF-I analog
8	C S R N L I D C	PDGF-BB peptide fragment
9	A G C K N F F W K T F T S C	Somatostatin-14
10	C H S G Y V G V R C	TGF $\alpha$ (34-43) (rat)
11	C I E L L Q A R C	Selectin binding peptide
12	C Y F Q N C P R G-NH <sub>2</sub>	[Arg8]-vasopressin
13	C Y I Q N C P L G-NH <sub>2</sub>	oxytocin

(arbitrary units). Data shown in this study were typically an average of 50 scans.

## Results and Discussion

### General ETD Phenomena

ETD data of peptide **1** ( $[M+2H]^{2+}$ , Figure 1a) and peptide **2** ( $[M+3H]^{3+}$ , Figure 1b) are used as examples to illustrate the typical fragments observed from a peptide containing an intrachain disulfide bond. Note that the peak assignments are based on comparing the observed masses to the ones predicted from the peptide sequence with the disulfide bond homolytically cleaved ( $\bullet$ sCXXCs $\bullet$ , XX: peptide backbone). However, fragments may not necessarily have the indicated structures. For example, a  $z$  ion does not have a biradical structure (one at thiol and the other one at  $\alpha$ -carbon) predicted from  $\bullet$ sCXXCs $\bullet$ . A series of  $c_n$  ( $n=4-6, 8-9$ ) and ( $n=7-9$ ) ions can be easily identified in Figure 1a. Ions with masses corresponding to  $c_n - 33$  Da ( $n=4, 6-7$ ),  $z_n + 33$  Da ( $n=7-9$ ),  $c_n + 32$  Da ( $n=4, 6, 9$ ),  $z_n - 32$  Da ( $n=7, 9$ ) are also observed. The presence of these types of ions with relatively high abundance has not been previously reported. Similar types of fragments were observed in ETD of  $[M+3H]^{3+}$  ions from peptide **2** (Figure 1b). In addition,  $c_n + 1$  Da ( $n=6-7$ ) ions were detected, clearly in higher abundance than their corresponding  $c_n$  ions, as shown in the insets of Figure 1b. Since peptides **1** and **2** have circular structures, the various types of backbone fragments can only be resulted from both backbone (N-C $\alpha$ ) bond and disulfide bond cleavages. Furthermore, these backbone fragments are likely resulted from a single electron transfer event,

given that doubly-charged peptide **1** was subjected to ETD, and the similar types of fragments were observed for the triply-charged ions with much shorter reaction time.

### Formation of $c$ , $z$ Ions

Cleavages at N-C $\alpha$  backbone bonds and disulfide bonds are competitive processes in ETD and ECD. Backbone N-C $\alpha$  bond cleavage gives rise to complementary pairs of  $c$  (even electron) and  $z^\bullet$  ions, while disulfide bond cleavage forms a sulfhydryl group ( $-SH$ ) and a thiyl radical ( $-S^\bullet$ ) at the cleavage site. Either cleavage may take place first, with the thus formed radical site initiates subsequent bond cleavages. It has been suggested from ECD studies on cyclic disulfide peptides that the N-C $\alpha$  bond is cleaved first to form a transient  $\alpha$ -carbon radical (the conventional  $z^\bullet$  species), which then attacks the disulfide bond, resulting in a cyclic  $z$  ion (even electron) and its complementary open-chain  $c^\bullet$  ion [13]. This process is summarized in Scheme 1, by using ETD of a triply-protonated peptide ion as an example. Given that only amide structure has been detected for  $c$  ions from ECD of charge-tagged peptides in IR ion spectroscopy and the amide structure is energetically more stable than its enole imine isomer, the  $c$ -type ion in Scheme 1 is drawn in the amide form [29]. The locations of the charge-bearing sites are not indicated in the scheme. In order to verify this hypothesis,  $c$  and  $z$  ions formed within the cyclic region of disulfide linked peptides were subjected to collisional activation. Figure 2a shows the CID spectrum of a typical  $z$  ion,  $z_5$  ( $m/z$  621.2, sequence: FLKKC) derived from ETD of triply-protonated peptide **3**. The peaks at  $m/z$  493.1 and 365.1 correspond to the loss of two successive lysine

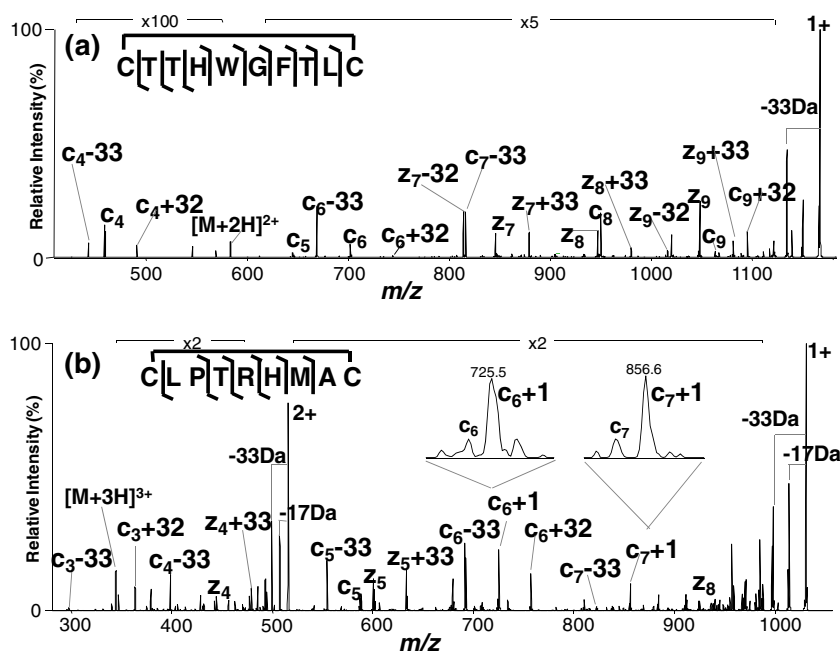
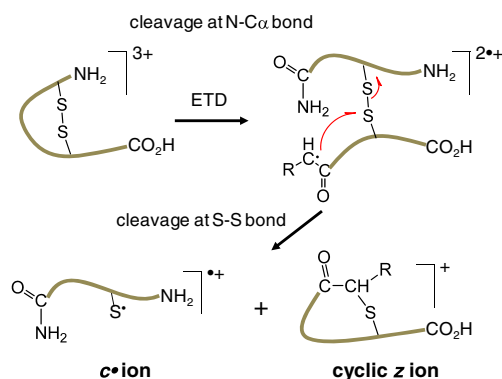


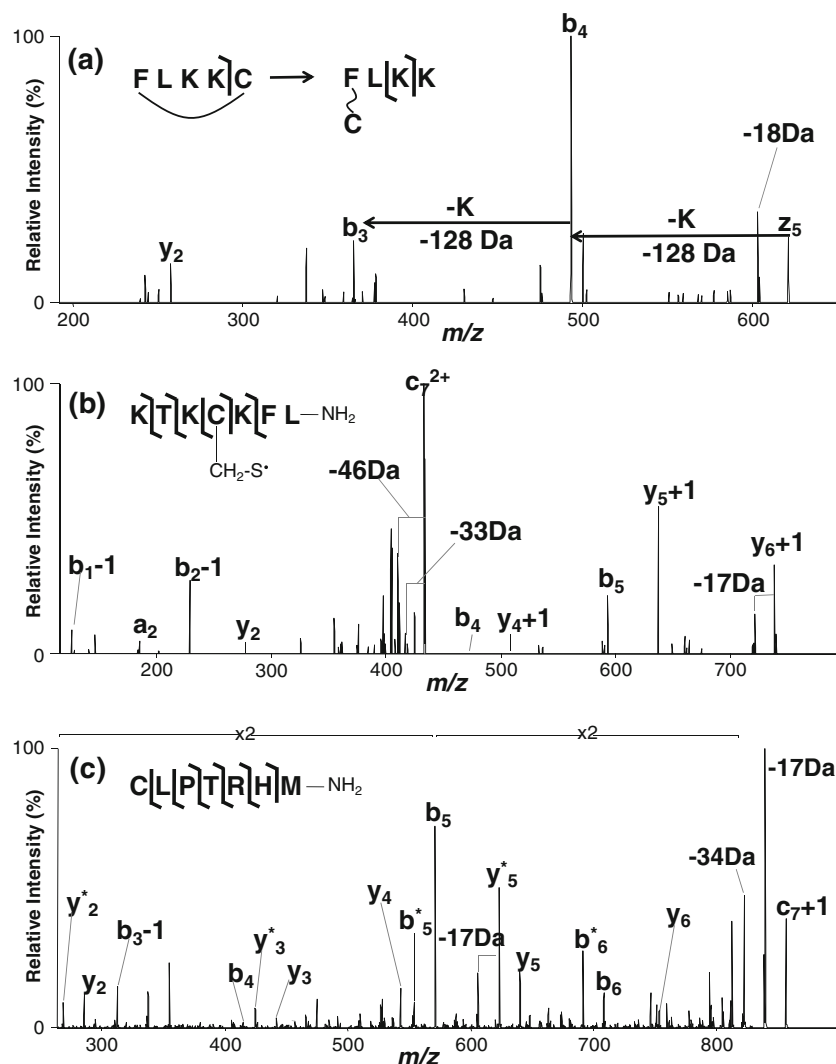
Figure 1. MS<sup>2</sup> ETD spectra resulted from (a)  $[M+2H]^{2+}$  ions of peptide **1** and (b)  $[M+3H]^{3+}$  ions of peptide **2**

Scheme 1. Formation of  $c^\bullet$  ion and cyclic  $z$  ion

residues (−128 Da) from the parent ion ( $z_5$ ). These fragments can only be generated with the  $z$  ion having a cyclic structure, where the cysteine (C) and phenylalanine (F) residues are initially connected. Upon collisional activation, the ring structure is opened up between the lysine (K) and

cysteine (C) residues, resulting in a new sequence, CFLKK. Subsequent amide bond cleavages generate  $y_2$ ,  $b_3$ , and  $b_4$  fragments, corroborating the mechanism proposed in Scheme 1 for the formation of cyclic z ions.

CID data of the doubly-charged  $c_7$  ( $m/z$  433.2) of peptide **3** are shown in Figure 2b. The detection of neutral losses, such as  $-33$  Da (SH) and  $-46$  Da ( $\text{CH}_2\text{S}$ ), are highly suggestive of the presence of a thiyl radical. Several b and y ions are observed, indicating a typical open-chain structure. Interestingly, the y ions all have masses 1 Da higher than expected (based on the c ion structure proposed in Scheme 1), in concert with their complementary b ions forming with masses 1 Da lower than expected ( $y_6+1/b_1-1$ ,  $y_5+1/b_2-1$ ). This observation indicates hydrogen transfer from the first two amino acid residues to the thiyl radical. In fact, experimental and theoretical studies have shown that intramolecular hydrogen transfer to the thiyl radical is a facile process within polypeptides and cysteine ions [30–33].



**Figure 2.** MS<sup>3</sup> CID of **(a)** z<sub>5</sub><sup>+</sup> ions derived from ETD of [M+3H]<sup>3+</sup> of peptide **3**, **(b)** c<sub>7</sub><sup>2+</sup> ions derived from ETD of [M+4H]<sup>4+</sup> of peptide **3**, and **(c)** (c<sub>7</sub>+1)<sup>+</sup> ions derived from ETD of [M+3H]<sup>3+</sup> of peptide **2**

CID of the  $c_7+1$  ( $m/z$  856.4, sequence: CLPTRHM) of peptide **2** (Figure 2c) clearly suggests an open-chain structure, with b and y ions appearing exactly at their predicted masses. The  $c+1$  ions are likely formed via the addition of a hydrogen to the thiyl radical at the cysteine residue, rather than at any other place along the sequence. Figure 2c also shows a high abundance of fragments for the loss of 17 Da ( $\text{NH}_3$ ) from parent ions, as well as from b/y fragments to form  $b^*/y^*$  ions. The abundant ammonia loss is generally observed from CID of c ions derived from ETD of linear peptides [34] or CID of N-terminus amidated peptides [35].

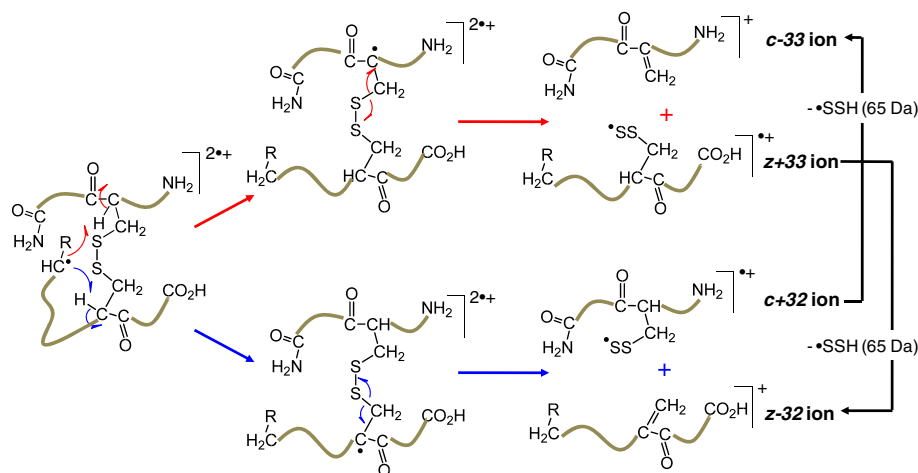
An alternative pathway of forming c and z ions within the cyclic region begins with an initial cleavage at the disulfide bond, followed by N-C $\alpha$  bond dissociation. If ETD cleaves the disulfide bond first, a thiyl radical ( $-\text{S}^\bullet$ ) and sulfhydryl group ( $-\text{SH}$ ) should be formed at the cleavage site [13]. An  $\alpha$ -carbon radical can be generated from radical migration [36], the rearrangement of which along the peptide backbone can induce N-C $\alpha$  cleavage as observed in ECD of circular peptide systems [37]. If these processes were at work in this study, complementary  $z+1$  and  $c-1$  ions would be formed. The fact that no  $z+1$  ions or  $c-1$  ions were observed in ETD spectra for any of the peptides examined in this study suggests that this mechanism is not likely to be a dominant process. In addition, collisional activation of peptide thiyl radical ions showed that the radical-initiated backbone cleavage (forming  $c-1$  and  $z+1$  ions) was sensitive to amino acid composition and only counted for a small fraction among a variety of fragmentation channels [38]. These results further suggest that even if N-C $\alpha$  bond cleavage were to occur after disulfide bond cleavage, its contribution to the observed backbone fragments should be quite small.

### Formation of $c-33/z+33$ and $c+32/z-32$ Ion Pairs

The  $c-33/z+33$  and  $c+32/z-32$  complementary ion pairs, which could only be resulted from both backbone N-C $\alpha$

dissociation and C-S bond cleavage within a cysteinyl side chain, were seen in relatively high abundance for ETD of peptides containing intrachain disulfide bonds. Although initial cleavage at C-S bond from ETD can happen, it is not likely that the resulting carbon-centered radical induces frequent N-C $\alpha$  backbone cleavages as suggested by studies on hydrogen deficient peptide radical ions [39]. A more plausible sequence is initial cleavage at the N-C $\alpha$  bond followed by C-S bond cleavage. Scheme 2 outlines the proposed mechanism for generation of  $c-33/z+33$  and  $c+32/z-32$  ion pairs. The formation of each ion pair can occur in a similar fashion, beginning with initial N-C $\alpha$  bond cleavage to form a transient  $\alpha$ -carbon radical. This radical site then migrates to the  $\alpha$ -carbon of either cysteine residue through intramolecular hydrogen transfer. A subsequent  $\beta$ -cleavage breaks the C-S bond, forming dehydroalanine and perthiyl radical ( $-\text{SS}^\bullet$ ). When the C-S bond is cleaved at the N-terminus cysteine side, a  $c-33/z+33$  ion pair is formed, while cleavage at the C-terminus cysteine side gives rise to a  $c+32/z-32$  ion pair.

Each type of fragment ion was subjected to CID to elucidate its structure. As suggested in Scheme 2,  $c-33$  and  $z-32$  ions are even electron species with the original cysteine residue modified to dehydroalanine. The CID spectrum of the  $c_7-33$  ion ( $m/z$  816.3, sequence: CTTHWGF) of peptide **1** (Figure 3a) indicates an open-chain structure with significant fragmentation to form b and y ions. All the b fragments have masses corresponding to a loss of 33 Da based on their sequence, while y fragments do not show any mass shifts, consistent with the proposed  $c-33$  ion structure. CID data of  $z_7-32$  ion ( $m/z$  814.2, sequence HWGFTLC) of peptide **1** are shown in Figure 3d. In this case, the cysteine residue at the C-terminus is modified to dehydroalanine. Accordingly, all b ions are observed without mass shifts, while y ions show a loss of 32 Da. The CID spectra of  $z_7+33$  ( $m/z$  879.2, sequence: HWGFTLC) and  $c_9+32$  ( $m/z$  1095.4, sequence: CTTHWGFTL) of peptide **1** are shown in Figure 3b and c,



Scheme 2. Formation of  $c-33/z+33$  and  $c+32/z-32$  ion pairs



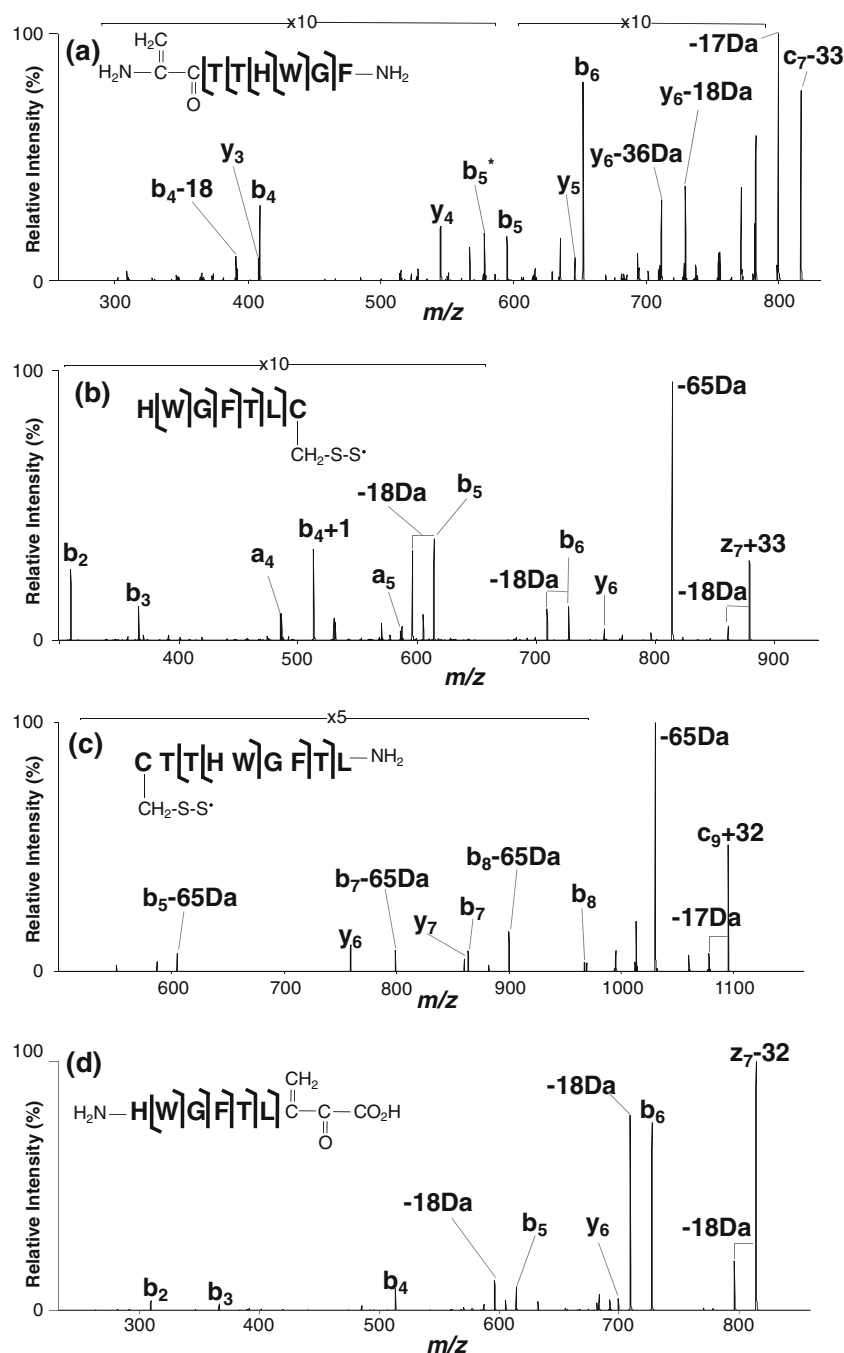


Figure 3. MS<sup>3</sup> CID of (a) c<sub>7</sub>-33 ion, (b) z<sub>7</sub>+33 ion, (c) c<sub>9</sub>+32 ion, and (d) z<sub>7</sub>-32 ion derived from ETD of [M+2H]<sup>2+</sup> of peptide 1

respectively. The most abundant peak is a loss of 65 Da (SSH) from parent ion in either case. Losses of 65 Da from b ions, e.g., b<sub>8</sub> - 65 (m/z 900.3), b<sub>7</sub> - 65 (m/z 799.3), and b<sub>5</sub> - 65 (m/z 595.3), are also evident in Figure 3c. This 65 Da loss can only be reasonably explained by having the radical site reside on the sulfur atom, corroborating the perthiyl radical (-SS<sup>•</sup>) structure. The b/y fragment masses exactly match the masses predicted from their structures, supporting the proposed structures of these ion species.

With the observation of these abundant losses of 65 Da in mind, it may be predicted that a competitive mechanism for

the formation of z - 32 and c - 33 ions also exists. This mechanism involves a perthiyl radical (-SS<sup>•</sup>) abstracting the α-carbon hydrogen within its own cysteine residue, resulting in a β-cleavage that liberates ·SSH, leaving the remaining piece modified to dehydroalanine. However, the formation of z+33 and c+32 ions can only be resulted from initial backbone cleavage followed by fragmentation at the C-S bond. The mechanisms proposed in Schemes 1 and 2 share a common feature, in that the N-Cα bond is the initial cleavage site in ETD and the subsequent radical-driven reactions cause either

S–S or C–S bond dissociation within cysteinyl residues. This pattern of events would yield all the ions present in high abundance in ETD spectra, accounting for the observation that N–C $\alpha$  and disulfide bond cleavage can arise from a single electron transfer event.

### Neutral Loss of 33 Da

Neutral loss of 33 Da ( $\bullet$ SH) from charge-reduced parent ions is widely observed for all peptides studied, although its relative abundance varies. The most probable pathway accounting for this loss involves cleavage first at the disulfide bond, forming a transient thiyl radical ( $-\text{S}\bullet$ ), and subsequent elimination of  $\bullet$ SH to form dehydroalanine. The 33 Da loss ions should have an open chain structure sharing the same sequence as the original peptide ions, with one cysteine residue being modified to dehydroalanine and the other to a sulfhydryl group. The CID data of  $([\text{M}+2\text{H}]^{++} - 33 \text{ Da})$  derived from ETD of doubly-protonated peptide **1** is shown in the Supporting Information (Figure S-1) and the observed fragments are consistent with the predicted ion structure. Unlike the  $c/z$ ,  $c - 33/z+33$  and  $c+32/z - 32$  ion pairs, which are formed from initial cleavage at N–C $\alpha$  bond followed by radical driven reactions, the 33 Da loss should only be resulted from direct ETD at the disulfide bond. Therefore, the relative intensity of this ion can be used to monitor the competition between disulfide bond cleavage and backbone N–C $\alpha$  cleavage in ETD of peptides containing intrachain disulfide bonds, as evidenced below in the “Effect of Charge State” section.

### Charge-Reduced Peptide Ions

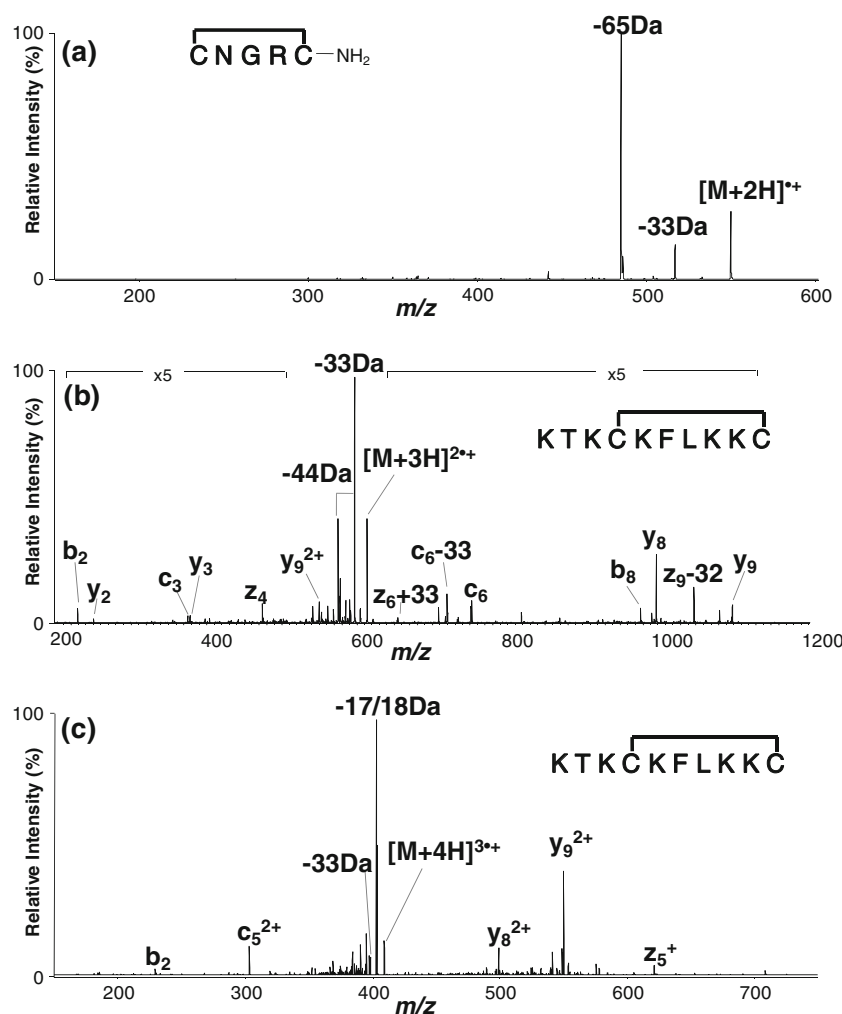
Charge-reduced peptide ions are commonly observed in ETD experiments, including both even-electron and odd-electron species. The charge-reduced, even-electron ions  $([\text{M}+(n-1)\text{H}]^{(n-1)+})$  are proposed to be formed either as a consequence of ejecting a hydrogen atom [40] or by competitive proton transfer reactions [41]. The radical peptide ions  $([\text{M}+n\text{H}]^{(n-1)+\bullet})$ , also referred to as electron transfer without dissociation (ETnoD) product, can be complexes consisting of non-covalently bonded  $c/z$  fragments [42] or intact peptide ions with significantly weakened N–C $\alpha$  bonds after electron transfer [43]. The ETnoD ions were abundantly observed for most of the peptides containing intrachain disulfide bonds herein. Note that due to the cyclic nature of the peptides, these ETnoD ions should still be in one piece after ETD cleavage at either the disulfide bond or an N–C $\alpha$  bond at the peptide backbone. The different structural isomers of the ETnoD products may explain the observation of several different fragmentation behaviors, as discussed below.

In general, neutral losses of 33 and 65 Da,  $c/z$ ,  $c - 33/z+33$ ,  $c+32/z - 32$ , and  $b/y$  ions were commonly observed from CID of ETnoD products. The neutral loss of 33 Da is

due to the loss of SH from the thiyl radical after ETD at the disulfide bond, while loss of 65 Da indicates the possibility of ETD at the C–S bond to form a perthiyl radical ( $-\text{SS}\bullet$ ). Interestingly, loss of 65 Da was not directly detected from ETD at a significant level, but was found to be abundant in CID of the charge-reduced ions in some cases as shown in Figure 4a. It was also observed that the neutral losses were typically more prominent in CID of lower charge states than higher charge states, where mobile protons were available (Figure 4a  $([\text{M}+2\text{H}]^{++})$  of peptide **5**) and 4b  $([\text{M}+3\text{H}]^{2++})$  of peptide **3**). The formation of  $c/z$ ,  $c - 33/z+33$ ,  $c+32/z - 32$  types of ions were attributed to the mechanisms depicted in Schemes 1 and 2. For CID of higher charged ETnoD ions (Figure 4c, CID of  $([\text{M}+4\text{H}]^{3++})$  derived from peptide **3**),  $b$  and  $y$  ions outside of the disulfide bond region ( $b_2^+$ ,  $y_8^{2+}$ , and  $y_9^{2+}$ ) were observed with significant abundances. The  $y_8^{2+}$  ion is a radical ion having a mass 1 Da higher than that is predicated from the sequence (with intact disulfide bond). The  $y_9^{2+}$  ion is 2 Da higher than the prediction, suggesting the cleavage of the disulfide bond and the formation of sulfhydryl at both cysteines. This observation is consistent with that its complementary fragment,  $b_2^+$ , is 1 Da less from the prediction. Given the difficulty of cleaving a disulfide bond under low energy CID conditions [2], the disulfide bond should be already cleaved from ETD and the charge directed fragmentation at amide bonds during CID finally gave rise to these  $b$  and  $y$  ions.

### Effect of Charge State

It is of interest to study the competition between cleavage at the disulfide bond and at an N–C $\alpha$  bond since several ECD and ETD studies reported preferential cleavages at disulfide bonds, especially for low charge states of peptides or proteins [13, 44]. The earlier discussion showed that ETD at the disulfide bond produces neutral loss such as 33 Da (SH), while the  $c/z$ ,  $c - 33/z+33$ ,  $c+32/z - 32$  fragment ions were formed from initial cleavage at N–C $\alpha$  bonds. Therefore, the relative intensity of the 33 Da neutral loss normalized to the sum of all fragments could be used to assess the degree of disulfide bond cleavage from an ETD event. Note that not all ETD products are readily in the fragment ion forms. Due to the cyclic structure of the peptides containing intrachain disulfide bonds, there is a certain fraction of peptide ions (ETnoD products) still in one piece but having a cleavage at either the disulfide bond or an N–C $\alpha$  bond. In order to include these ions into consideration, supplementary activation (SA) during ETD was used to dissociate at around 90% of the first generation ETnoD products. Figure 5 shows the data collected for ETD of quadruply-, triply-, and doubly-protonated ions of peptide **3** with SA applied. The insets in Figure 5 compare the expanded regions of the first generation ETnoD products with and without SA. Clearly, the ETnoD radical ions were predominantly formed relative to the even electron species from ETD. After SA, more than 90% of the radical ions



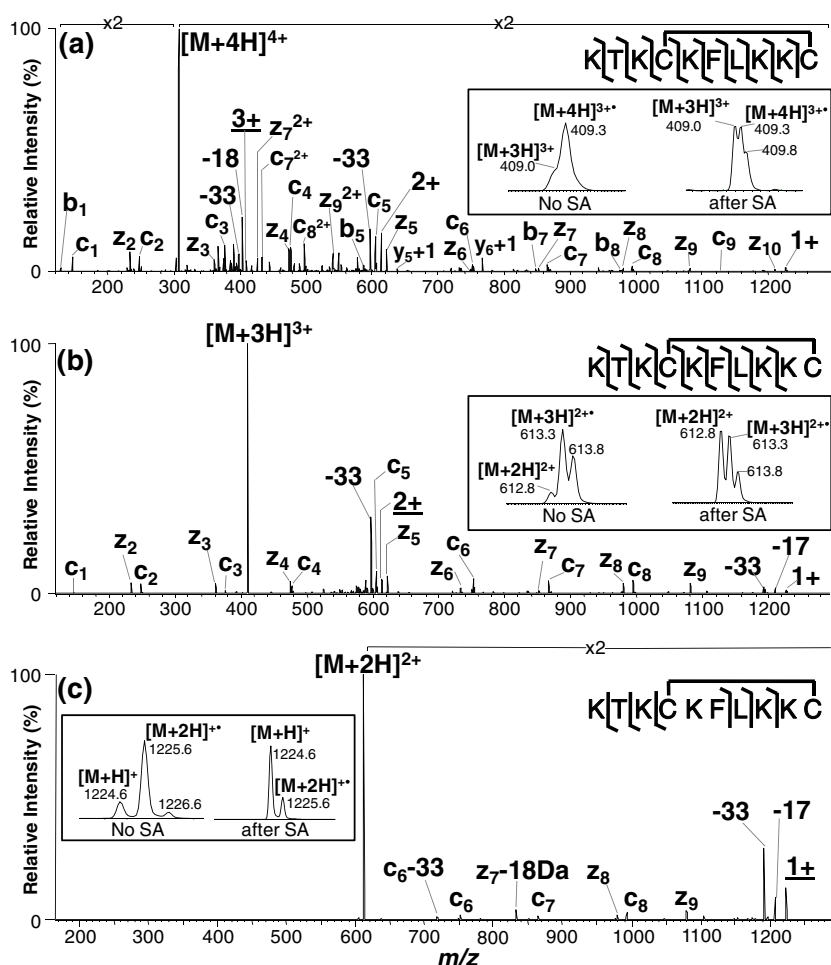
**Figure 4.** MS<sup>3</sup> CID of first generation charge reduced peptide ions derived from ETD: (a)  $[M+2H]^{2+}$  of peptide 5, (b)  $[M+3H]^{3+}$  of peptide 3, and (c)  $[M+4H]^{4+}$  of peptide 3

were dissociated while the even electron ions were left unchanged. When comparing the ETD data with SA to that of no SA (Figure 5 to Figure S-2), it is worth pointing out that the backbone fragmentation patterns were almost identical for all charge states (except that a couple of new backbone fragments are observed from ETD of 2+ with SA). On the other hand, a dramatic increase of signals due to neutral loss of 33 Da for all charge states was observed when SA was applied. The increase of 33 Da loss simply indicates that a significant fraction of ETnoD ions undergo direct cleavage at the disulfide bond. The fractions of the neutral loss 33 Da from first generation ETnoD products normalized to the sum of all fragment ion intensities were calculated and were found to be  $3.2\% \pm 0.1\%$ ,  $12.7\% \pm 0.3\%$ , and  $34.9\% \pm 0.3\%$  for ETD of 4+, 3+, and 2+ charge state, respectively. These values were calculated based on at least three separate data collections, with standard deviations indicated. There is a clear trend of increased fragmentation at the disulfide bond as the charged state decreases. This trend is consistently observed for peptides 2, 6, 7, and 9, as well. (Supporting Information, Table S-2).

### Implications to Peptide Sequencing

A common feature shared among all the peptides investigated in this study is that the entire or the majority of the peptide backbone is confined within the ring structure, due to the formation of an intrachain disulfide bond. Obtaining sequence information for this type of peptide is very challenging with the conventional CID method. This is because two bonds need to be cleaved to form one sequence ion, which is not always accessible from low energy CID conditions. ETD, as discussed above, can induce both N-C $\alpha$  backbone cleavage and disulfide bond cleavage in a single electron transfer event, allowing the observation of sequence ions within the cyclic region. Table S-1 in the Supporting Information summarizes the observed major fragments resulting from ETD for 13 peptides containing intrachain disulfide bonds. The sites of fragmentation are indicated along the peptide sequence, based on the observed c/z ions. Rich sequence information can be obtained from all peptides. The extent of cleavage corresponds to 75%–100% of possible backbone fragmentation, which is highly desirable from the perspective of peptide sequencing. The detected c-33,





**Figure 5.** ETD with simultaneous supplementary activation (SA) (a)  $[M+4H]^{4+}$ , (b)  $[M+3H]^{3+}$ , and (c)  $[M+2H]^{2+}$  ions derived from peptide 3. The insets are expanded view of the first generation ETnOD product ion region with or without SA. With SA applied, more than 80% of the ETnOD product ions are dissociated

$z+33$ ,  $c+32$ , and  $z-32$  ions are listed in separate columns in Table S-1. These types of ions were observed in many cases and were typically more prevalent in ETD of lower charge states of peptide ions. Including these types of ions in the generation of in-silico peptide fragmentation should be beneficial to the identification of disulfide-linked peptides. In addition, the presence of these ions is unique to fragments formed within the cyclic backbone region confined by a disulfide bond, which can be potentially used to confirm the location of the disulfide bond.

## Conclusions

Electron transfer dissociation (ETD) was shown to induce both disulfide bond cleavage and backbone fragmentation from a single electron transfer event, allowing for sequence information to be obtained from a region of peptide backbone cyclized by intrachain disulfide bonds. In addition to the  $c/z$  ions typically observed in ETD, new types of fragments corresponding to complementary ion pairs of  $c-33/z+33$  and  $c+32/z-32$  were identified. Mechanistic

studies suggested that the formation of these ions involved initial ETD at N-C $\alpha$  backbone followed by radical-driven reactions at cysteinyl residues. A competing fragmentation channel associated with ETD at the disulfide bond was detected, with a signature loss of 33 Da (SH) from the charge-reduced peptide ions. Similar to reports on ETD of linear peptides, reduced fragmentation was observed for ETD of the lower charge states of peptide ions. However, for the 13 peptides investigated herein, extensive backbone fragmentation was typically observed from ETD, suggesting that ETD could be very useful in providing sequence information for peptides containing intact intrachain disulfide bonds. Given the frequent appearance of  $c-33/z+33$  and  $c+32/z-32$  ion pairs in ETD, including these ions in sequencing should allow enhanced identification of proteins containing intrachain disulfide bonds.

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