Available online at www.sciencedirect.com
ScienceDirect
International Journal of Mass Spectrometry 263 (2007) 71–81
International Journal of Mass Spectrometry
www.elsevier.com/locate/ijms

Preferential cleavage of S–S and C–S bonds in electron detachment dissociation and infrared multiphoton dissociation of disulfide-linked peptide anions

Anastasia Kalli, Kristina Håkansson *

Department of Chemistry, University of Michigan, 930 North University Avenue, Ann Arbor, MI 48109-1055, USA

Received 22 November 2006; accepted 2 January 2007
Available online 7 January 2007

Abstract

Disulfide bonds generally show only limited cleavage in positive ion mode collision activated dissociation (CAD). However, it has been demonstrated that a reverse situation exists in negative ion mode in which preferential S–S and C–S bond cleavage occurs. Here, we show that electron detachment dissociation (EDD) and infrared multiphoton dissociation (IRMPD) of peptide anions containing disulfide linkages also result in preferential cleavage of S–S and C–S bonds. Resulting products are mainly radical ions in EDD whereas IRMPD produces even-electron product ions, as expected, thereby supporting different disulfide cleavage mechanisms for these two fragmentation processes. We also show that, in EDD, the presence of tryptophan can result in abundant side chain loss (129 Da), which effectively can compete with disulfide bond cleavage.

Keywords: Negative ion mode electrospray ionization; Disulfide bond; Fourier transform mass spectrometry; Electron detachment dissociation; Infrared multiphoton dissociation

1. Introduction

Tandem mass spectrometry (MS/MS) [1] is widely used for peptide sequencing and protein characterization [2–7]. Several dissociation techniques, including collision activated dissociation (CAD) [8,9], infrared multiphoton dissociation (IRMPD) [10,11], surface induced dissociation (SID) [12–14] UV photodissociation [15,16], electron capture dissociation (ECD) [17], electron detachment dissociation (EDD) [18], and electron transfer dissociation (ETD) [19], have been employed for peptide ion fragmentation. Each technique has unique advantages and disadvantages and the mode of fragmentation needs to be carefully chosen to suit a particular application. For a protein to be completely characterized, identification and localization of post-translational modifications (PTMs) such as phosphorylation, glycosylation, acetylation, methylation, sulfation, and disulfide bond formation, is essential. That is because such modifications can determine protein function and activity as well as interactions with other molecules. Disulfide bond formation is a PTM occurring in extracellular proteins. Determination of the presence and connectivity of disulfide bonds is of great interest due to their importance for the stabilization of the native structures of proteins [20–23]. Thus, knowledge considering disulfide bridging is a significant component for a complete understanding of the chemical structure and folding of a protein [23,24].

Traditionally, determination of disulfide linkages by mass spectrometry is performed by comparing spectra of proteolytic peptides obtained with and without reduction and alkylation of the disulfide bonds. MS/MS is an alternative approach, offering the advantage of decreased analysis time. However, low energy CAD of cations, which is by far the most widely used fragmentation strategy, yields little cleavage of disulfide bonds. Thus, alternative dissociation methods are needed. Disulfide bonds can cleave in high energy CAD [25] and in matrix-assisted laser desorption/ionization post-source decay (MALDI-PSD) [26], which also involves high energy deposition. In addition, McLafferty and co-workers demonstrated that ECD, which involves gas-phase cation radical chemistry, preferentially cleaves disulfide bonds [27]. Zubarev and co-workers have noted similarities between ECD and MALDI in-source decay (ISD) in terms of peptide backbone cleavage and retention of PTMs and proposed that ISD is mediated by hydrogen radicals [28]. Another sim-

* Corresponding author. Tel.: +1 734 615 0570; fax: +1 734 647 4865.
E-mail address: kicki@umich.edu (K. Håkansson).

1387-3806/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijms.2007.01.001

ilarity is indeed the facile cleavage of disulfide bonds that has been reported in MALDI ISD [29,30]. Furthermore, Fung et al. recently demonstrated that photodissociation at 157 nm provides cleavage of both intra and intermolecular disulfide bonds in peptides and proteins [31]. That process was proposed to occur via electronic excitation and radical intermediates. Finally, ETD, which involves electron transfer from small anions and in many ways is analogous to ECD, also provides disulfide bond cleavage in peptide cations [32].

Chrisman and McLuckey have demonstrated that CAD of peptide anions containing disulfide bridges results in preferential cleavage of S–S and C–S bonds [33]. This strategy was recently extended by Zhang and Kaltashov [34]. In addition, Standing and co-workers showed that the singly charged anion of insulin decays into its constituent A and B chains, involving cleavage of two intermolecular disulfide bonds, following MALDI [35]. Although not nearly as extensively used as positive ion mode for protein analysis, negative ion mode can provide complementary information and is particularly valuable for acidic proteins [36], constituting ~50% of the protein pool, and for characterization of acidic PTMs, such as phosphorylation [37] and sulfation [38]. For example Bigwarfe and Wood reported negative ion mode detection of a number of tryptic peptides that were not observed in positive mode [39]. They also highlighted the importance of employing both positive and negative ion mode for the improvement of protein sequence coverage and identification. In addition, Cassady and co-workers have shown that positive and negative ion PSD provide complementary information on peptide primary structure [40]. Fragmentation pathways of deprotonated peptides are not as well understood as those of protonated peptides. However, Bowie et al. have employed a combination of experiments and theory to investigate CAD dissociation pathways of singly deprotonated peptide ions [41]. That approach was recently applied to peptides containing an intramolecular disulfide bridge [42].

EDD is a rather recently introduced technique for dissociation of negative ions via a radical ion intermediate [18]. This technique has been shown to be analogous to ECD in that more random peptide backbone cleavage can be obtained compared to CAD [43] and that PTMs [18,44,45] as well as higher order structure [46] can be retained. In EDD precursor ions are irradiated with >10 eV electrons, resulting in electron detachment and subsequent fragmentation. A major fragmentation pathway in EDD of peptides is cleavage of backbone C_α–C bonds, forming x- and $a^\bullet$-type product ions [18,44]. Cleavage of N–C_α bonds, producing c and $z^\bullet$ ions, is also observed as well as y-type product ions [18,43]. The details of the EDD fragmentation process are still a matter of investigation, however, Zubarev and co-workers proposed that cleavage occurs near the location of negative charges [44]. Thus, EDD backbone cleavage should occur near acidic residues. Following the C-terminus and the side chains of aspartic and glutamic acid, the next most acidic sites in peptides are backbone amide nitrogens [44,47] and a unidirectional mechanism in which the radical sites are located on amide nitrogens has been proposed for the formation of x and $a^\bullet$ ions [44].

Here, we employ EDD and IRMPD in an electrospray ionization quadrupole-Fourier transform ion cyclotron resonance (ESI-Q-FTICR) mass spectrometer to characterize disulfide-bonded peptide anions. One goal of these experiments was to investigate whether EDD can be a useful technique for probing disulfide bonds in proteins. IRMPD is preferred over CAD in the cell of an FT-ICR instrument because there is no need for the introduction of collision gas, which deteriorates instrument performance and adds analysis time [48,49]. Because IRMPD and CAD are similar activation methods (both are based on slow heating of ions), we expected to observe disulfide bond cleavage in negative mode IRMPD. However, to our knowledge, such experiments have not previously been reported.

2. Methods

2.1. Sample preparation

Chicken egg white lysozyme and insulin from bovine pancreas were purchased from Sigma (St. Louis, MO). A 3.1 nmol of lysozyme were digested with trypsin (Princeton Separations, Aldelphia, NJ) at a 1:20 enzyme to protein ratio for 10 h at 37 °C. Insulin (1.8 nmol) was digested with Glu-C (Roche, Indianapolis, IN) at 1:20 or 1:40 enzyme to protein ratios for 9 h at 25 °C. The reactions were quenched with 0.1% formic acid (Acros Organics, Morris Plains, NJ). For lysozyme, the digested samples were desalted with C18 Ziptips (Millipore, Billerica, MA). For peptide pairs containing one intermolecular disulfide bond, improved recovery was obtained from the remaining solution after desalting, therefore that solution was used. The solution was diluted with 300 μL electrospray solvent containing 40% 2-propanol and 10 mM ammonium bicarbonate (Fisher Scientific, Fair Lawn, NJ). For peptide pairs containing one intermolecular and one intramolecular disulfide bond, the desalted solution was used after dilution with 600 μL spraying solvent, containing 40% 2-propanol and 10 mM ammonium bicarbonate or 20 mM tripropylamine. For insulin, no desalting was performed following digestion. The digested samples were diluted to a final concentration of 1.8 μM in 1 mL spraying solvent containing 40% 2-propanol and 5 mM ammonium acetate (Fisher).

The peptides p-Glu-Gln-D-Trp-Phe-D-Trp-D-Trp-Met-NH_2, H-Trp-His-Trp-Leu-Gln-Leu-OH, H-Gly-Asn-Leu-Trp-Ala-Tyr-Gly-His-Phe-Met-NH_2, and p-Glu-Val-Asn-Phe-Ser-Pro-Gly-Trp-Gly-Tyr-NH_2 were obtained from Sigma and the peptide H-Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-NH_2 was obtained from Advanced Chemtech (Louisville, KY) and used without further purification. The first two peptides were electrosprayed at 10 μM, and the three latter ones were electrosprayed at 5 μM from a spraying solvent containing 50% 2-propanol and 10 mM ammonium acetate.

2.2. Fourier transform ion cyclotron resonance mass spectrometry

All experiments were performed with an actively shielded 7 T Q-FT-ICR mass spectrometer (Apex-Q, Bruker Daltonics,

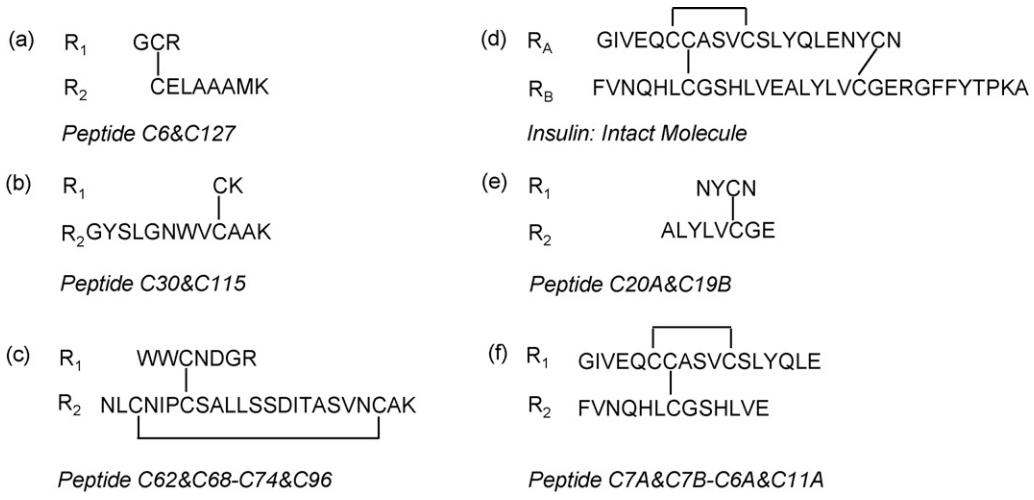
Billerica MA), which has been previously described [46]. The proteolytic mixtures were electrosprayed in negative ion mode at a flow rate of 80 μL/h, and the peptides were electrosprayed at a flow rate of 70 μL/h. Ions were accumulated in the first hexapole for 0.1 s, transferred through the mass selective quadrupole (10 *m/z* isolation window) and mass selectively accumulated in the second hexapole for 1–5 s for IRMPD experiments and 1–6 s for EDD experiments. All spectra were acquired with XMASS (version 6.1, Bruker Daltonics) using 256K or 512K data points and summed over 16–64 scans. Data processing was performed with the MIDAS analysis software [50]. EDD spectra were internally calibrated using the calculated masses of the precursor ion and the charge reduced species. IRMPD spectra were externally calibrated (see below), except for the peptide C6&C127 (Scheme 1a) for which internal calibration was performed using the calculated masses of the precursor and the $[M-H]^-$ ions. Oxidized insulin chain A (Sigma) was used as the external calibrant for the peptides C30&C115 (Scheme 1b), C62&C68-C74&C96 (Scheme 1c), C7A&C7B–C6A&C11A (Scheme 1f) and for the intact molecule of insulin (Scheme 1d). The pp60C-SRC carboxy-terminal phosphoregulatory peptide (Advanced Chemtech) was used as the external calibrant for the peptide C20A&C19B (Scheme 1e). Specifically, the b_6 ($m/z = 676.2617$) and y_{11} ($m/z = 1443.5049$) product ions from oxidized insulin chain A were used for the peptide C30&C115, the $[M-2H]^{2-}$ ($m/z = 1263.9491$) and b_8 ($m/z = 898.2928$) ions were used for the peptide C62&C68–C74&C96, the b_8 and y_{13} ($m/z = 1629.6054$) ions were used for the peptide C7A&C7B–C6A&C11A, and the b_{10} ($m/z = 1084.3933$) and y_{13} ions were used for insulin For the peptide C20A&C19B, the b_8 ($m/z = 933.3961$) and y_5 ($m/z = 527.2471$) product ions from pp60C-SRC carboxy-terminal phosphoregulatory peptide were used for calibration. Only peak assignments with a mass accuracy better than 15 ppm were accepted.

IRMPD experiments were performed with a vertically mounted 25 W, 10.6 μm, CO_2 laser (Synrad, Mukilteo, WA). Photon irradiation was performed for 0.05–0.08 s at 40–45% laser power, except in one case, peptide C6&C127 (see below), for which the photon irradiation was performed at 30% laser power. EDD experiments were performed with an indirectly heated hollow dispenser cathode at a bias voltage of 18–19.2 V and an irradiation time of 0.4–1 s. A lens electrode located in front of the hollow cathode was kept at 19–20 V.

3. Results and discussion

Lysozyme contains four disulfide bridges between cysteine 6 and cysteine 127, cysteine 30 and cysteine 115, cysteine 64 and cysteine 80, and between cysteine 76 and cysteine 94 [51]. Following proteolytic digestion with trypsin, three disulfide bond-containing species were detected with negative ion mode electrospray ionization. Two of these consisted of two peptide chains connected via an intermolecular disulfide bond; the peptide GCR disulfide-linked to the peptide CELAAAMK, here abbreviated as C6&C127 (Scheme 1a), and the peptide CK disulfide-linked to GYSLGNWVCAAK, here abbreviated as C30&C115 (Scheme 1b). We will refer to the sequences GCR and CK as R_1 and to the sequences CELAAAMK and GYSLGNWVCAAK as R_2 (Scheme 1a and b). The third species contains an additional intramolecular disulfide bond in one of the peptides; WWCNDGR (R_1) disulfide-linked to NLCNIPCSALLSSDITASVNCAK (R_2,) with an intramolecular disulfide bond between C3 and C21 of the R_2 peptide chain (Scheme 1c). This species will be referred to as C62&C68–C74&C96.

Insulin contains two polypeptide chains linked together with two intermolecular disulfide bridges between cysteine 7 of chain A and cysteine 7 of chain B and between cysteine 20 of chain A and cysteine 19 of chain B. In addition to these intermolecular disulfide bridges, insulin contains one intramolecular disulfide bond formed between cysteine 6 and cysteine 11 of chain A (Scheme 1d) [52,53]. Proteolytic digestion of insulin with Glu-C resulted in detection of two disulfide bond-containing species in negative ion mode. The first species contained one intermolecular disulfide bond: the peptide NYCN of chain A, disulfide-linked with the peptide ALYLVCGE of chain B, here referred to as C20A&C19B (Scheme 1e). The second species



Scheme 1. Structures of disulfide-bonded peptide pairs characterized by negative ion mode IRMPD and EDD.

contained one intermolecular disulfide bond and one intramolecular disulfide bond: the peptide GIVEQCCASVCSLYQLE of chain A disulfide-linked to the peptide FVNQHLCGSHLVE of chain B, here referred to as C7A&C7B–C6A&C11A (Scheme 1f).

3.1. EDD and IRMPD of peptide pairs containing one intermolecular disulfide bond

3.1.1. C6&C127

Fig. 1a shows the EDD tandem mass spectrum of the doubly deprotonated ($[M-2H]^{2-}$) ions of the peptide pair C6&C127 (Scheme 1a). The dominant product ions correspond to cleavage of either the intermolecular S–S bond or its neighboring C–S bonds, resulting in product ions containing none, one, or two sulfur atoms, respectively (see Scheme 2 for the product ion nomenclature). No product ions corresponding to peptide backbone cleavage are observed although neutral loss of CO_2 is seen, which constitutes a ubiquitous fragmentation channel in EDD [18,43]. This behavior can be rationalized within the previously proposed mechanism for EDD peptide fragmentation, depicting cleavage close to sites of deprotonation [44] because the R_2 peptide chain contains a glutamic acid residue next to the disulfide bond. The previously proposed cleavage mechanism also involves radical migration to the complementary fragment, consistent with the observation of primarily even-electron fragments containing the R_2 peptide chain. The only exception is the larger product $[R_2SSH-H]^-$, which also appears in its radical form $[R_2SS^{\bullet}-H]^-$, see inset. However, that product could be a result of electron detachment from a deprotonated R_1 C-

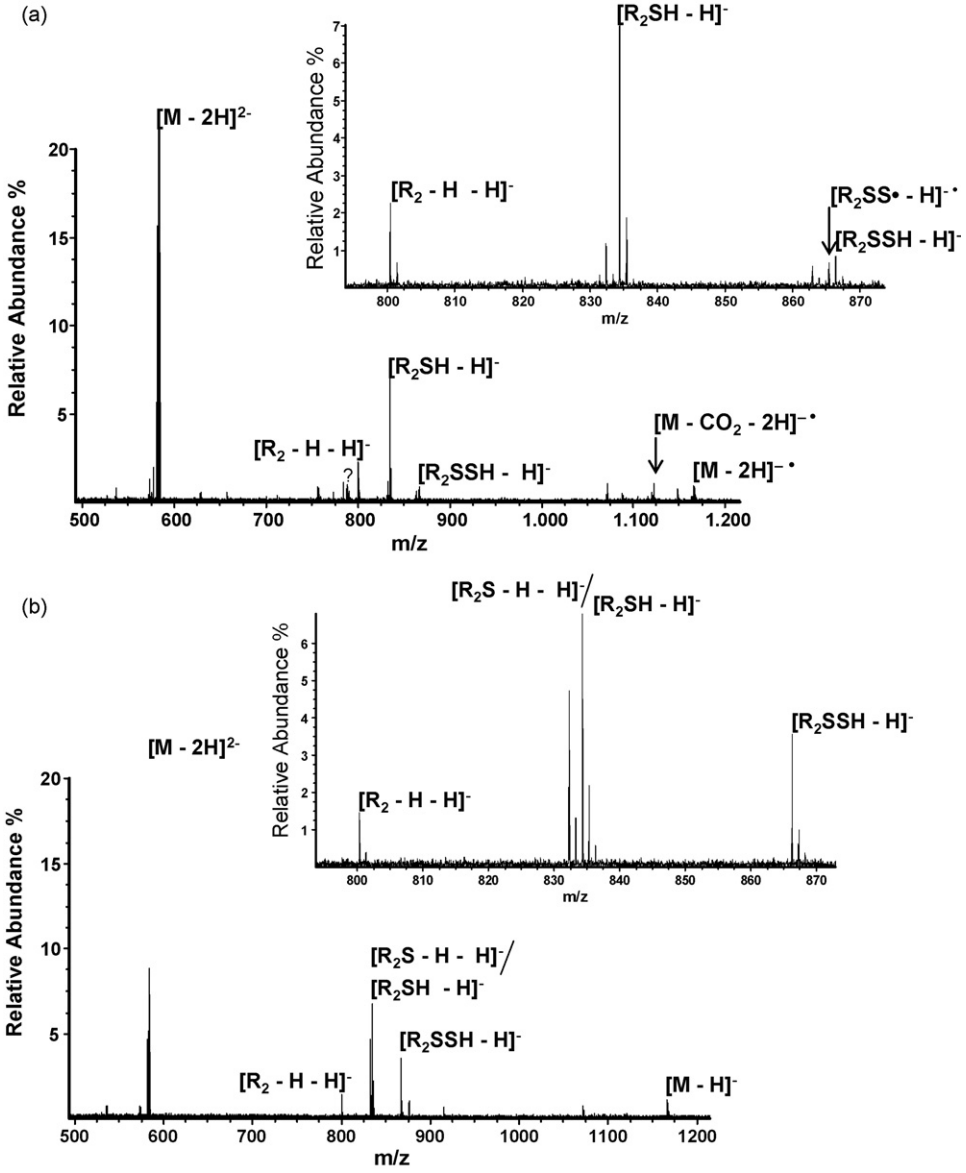
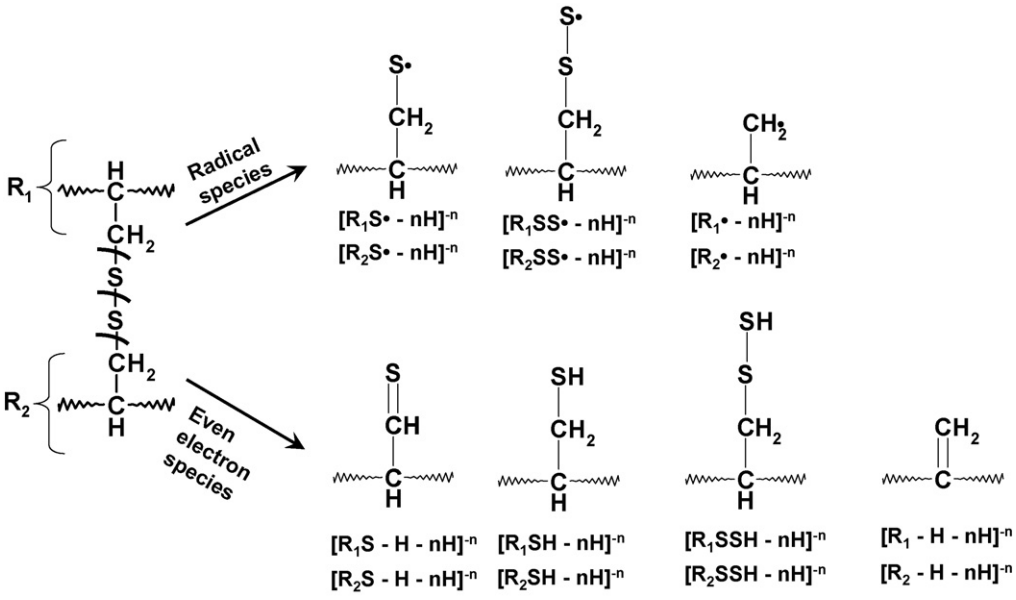


Fig. 1. EDD (a) and IRMPD (b) of the doubly deprotonated ions of the peptide pair C6&C127. One radical and two even-electron products are detected in EDD whereas solely even-electron species are observed in IRMPD. In some cases (e.g., insets), mixtures of product ions in which hydrogen atoms were transferred between the two chains, R_1 and R_2, are observed (see Scheme 2 for product ion structures and nomenclature). (?) Unidentified product ion.



Scheme 2. Structures and nomenclature used for the product ions observed in IRMPD and EDD.

terminus, also located in close proximity to the disulfide bond. Nevertheless, detection of this radical product ion implies that the disulfide cleavage mechanism in EDD is different from the one in negative ion mode CAD, which also results in prominent S–S and C–S bond cleavage [33]. We have previously noted that doubly charged product ions are observed in EDD of doubly charged precursor ions [46] and, furthermore, Zubarev and co-workers have demonstrated that extensive fragmentation of *singly* charged precursors occurs from ~10 eV electron irradiation [54]. In both cases, several observed product ions are of the same type as those observed in CAD/IRMPD and are believed to be a consequence of concomitant precursor ion vibrational excitation. Thus, one can envision that the observed disulfide cleavage in EDD originates from such a process but, because we observe radical product ions, we believe the cleavage mechanism is different than that in CAD/IRMPD.

To further explore whether EDD cleaves disulfide bonds in a manner different from vibrational excitation, we compared the EDD data to IRMPD of the same species. The corresponding spectrum for the peptide pair C6&C127 is shown in Fig. 1b. As for EDD and previous CAD, S–S and C–S bond cleavage dominate although no radical ions are observed from IRMPD, as expected. The inset in Fig. 1b reveals that the dominant product ion is a mixture of two species, $[R_2SH - H]^-$ and $[R_2S - 2H]^-$ (see Scheme 2 for nomenclature), where the former is somewhat more dominant. This mixture was also observed in EDD although the latter species was less abundant compared to IRMPD (Fig. 1a inset). CO_2 loss is not observed in IRMPD, further corroborating a cleavage mechanism different from that in EDD. In ECD of disulfide-bonded peptide cations a mixture of odd-electron $RS^\bullet$ and even-electron RSH products is also observed [27]. Cleavage of S–S bonds is highly favored over the backbone amine bond cleavage typically observed in ECD. One difference between ECD and EDD of disulfide-bonded peptides is the lack of backbone product ions in EDD, which could be a result of the low EDD fragmentation efficiency [48]. An alternative explanation is that electron detachment from disulfide bonds is favored over the previously proposed detachment from deprotonated acidic residues and deprotonated backbone amide hydrogens [18,44]. Indeed, disulfide bonds are easily oxidized to form radical cations [55]. The vertical ionization energy (IE) has been measured to be 8.46–9.1 eV [56], i.e., significantly lower than the electron energy used here (18–19.2 eV). This possibility will be further discussed below.

3.1.2. C30&C115 and C20A&C19B

Further information on the EDD behavior of disulfide-linked peptide pairs is gained from Fig. 2a, which shows EDD of the peptides C30&C115 (Scheme 1b). Here, radical product ions dominate (see inset), consistent with the hypothesis above, that electron detachment occurs from the proximal C-terminus of the R_1 peptide chain, leaving the radical site on the R_2 chain. As for the peptide C6&C127 (Fig. 1a), CO_2 loss is observed but to a lower extent than disulfide bond cleavage. IRMPD of C30&C115 (Fig. 2b) was very similar to IRMPD of C6&C127 in that disulfide bond cleavage to form even-electron product ions dominates. However, only $[R_2 - 2H]^-$ and $[R_2S - 2H]^-$ ions are observed in this case. A third example is given in Fig. 3a, which shows EDD of the peptide C20A&C19B (Scheme 1e). Again, S–S and C–S bond cleavages as well as CO_2 loss constitute the only observable fragmentation pathways. However, for this peptide pair, products containing both the R_1 and R_2 peptide chains are detected. The R_1 C-terminus is closer to the disulfide bond than the R_2 C-terminus. Consistently, the R_1-containing product ion is primarily an even-electron species whereas the R_2-containing products are predominantly radical species. IRMPD of C20A&C19B (Fig. 3b) also provided product ions containing both the R_1 and R_2 peptide chains. In addition, neutral losses of water and ammonia are seen as well as a neutral loss of 129 Da. The latter product most likely corresponds to loss of the R_2 C-

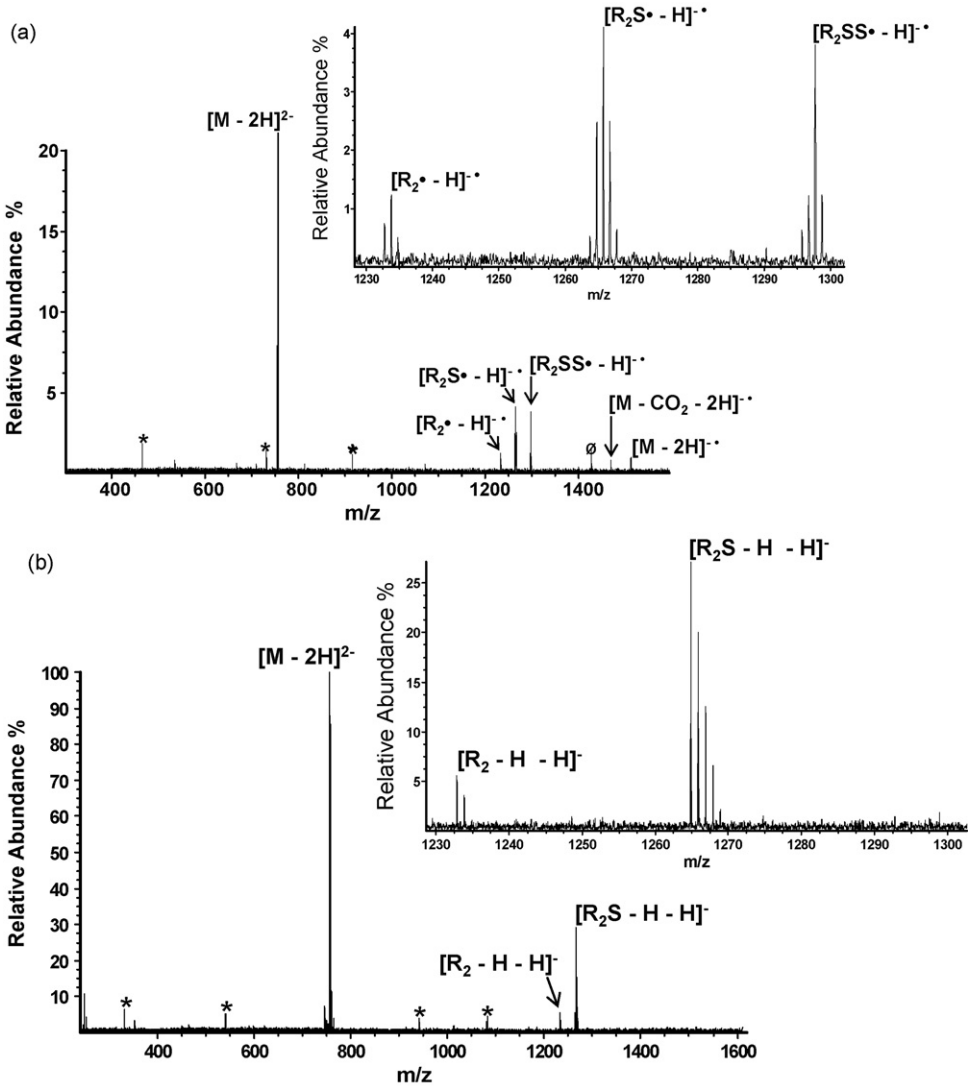


Fig. 2. EDD (a) and IRMPD (b) of the peptide pair C30&C115. As for C6&C127 (Fig. 1a), EDD resulted in cleavage of both S—S and C—S bonds. However, here, radical product ions are mainly observed whereas IRMPD again resulted in formation of even-electron species, supporting a cleavage mechanism different from that in EDD. The R_2 chain containing two sulfur atoms was not observed in IRMPD whereas this product ion was present as an odd electron species following EDD. (*) noise peaks.

terminal glutamic acid residue through a rearrangement reaction in which one of the carboxyl oxygens is retained, i.e., formation of a [b + H_2O] ion, as previously reported for protonated peptides [57–59].

3.2. EDD and IRMPD of peptide pairs containing one intermolecular and one intramolecular disulfide bond

3.2.1. C7A&C7B–C6A&C11A

The EDD mass spectrum of the $[M-3H]^{3-}$ ions of the peptide pair C7A&C7B–C6A&C11A is shown in Fig. 4a. Compared to the peptides discussed above, this peptide pair contains an additional intramolecular disulfide bond in the R_1 chain (see Scheme 1f). The EDD spectrum is dominated by the charge-reduced radical species, $[M-3H]^{2-\bullet}$. Charge reduction as a dominant process has previously been observed in EDD of nucleic acid anions and it has been proposed to be due to retention of hydrogen bonding, which prevents product ions from separating [46,60], similar to the behavior found in ECD [61]. Here, cleavage of the intramolecular disulfide bond would result in product ion pairs that are covalently linked, rendering it even more difficult to produce separated (and hence detectable) product ions. However, cleavage of the intermolecular disulfide bond should result in detectable product ions, which are observed although their relative abundance is lower than for peptide pairs lacking an intramolecular disulfide (Figs. 1–3). Interestingly, only product ions corresponding to S—S cleavage are observed whereas products corresponding to C—S bond cleavages are absent. However, the former products were more abundant for all peptide pairs discussed above (Figs. 1–3) so the absence of C—S cleavages could simply be a signal-to-noise (S/N) issue. Consistent with competition between intra- and intermolecular disulfide bond cleavage, the product ion corresponding to neutral CO_2 loss (whose detection is independent of disulfide cleavage)

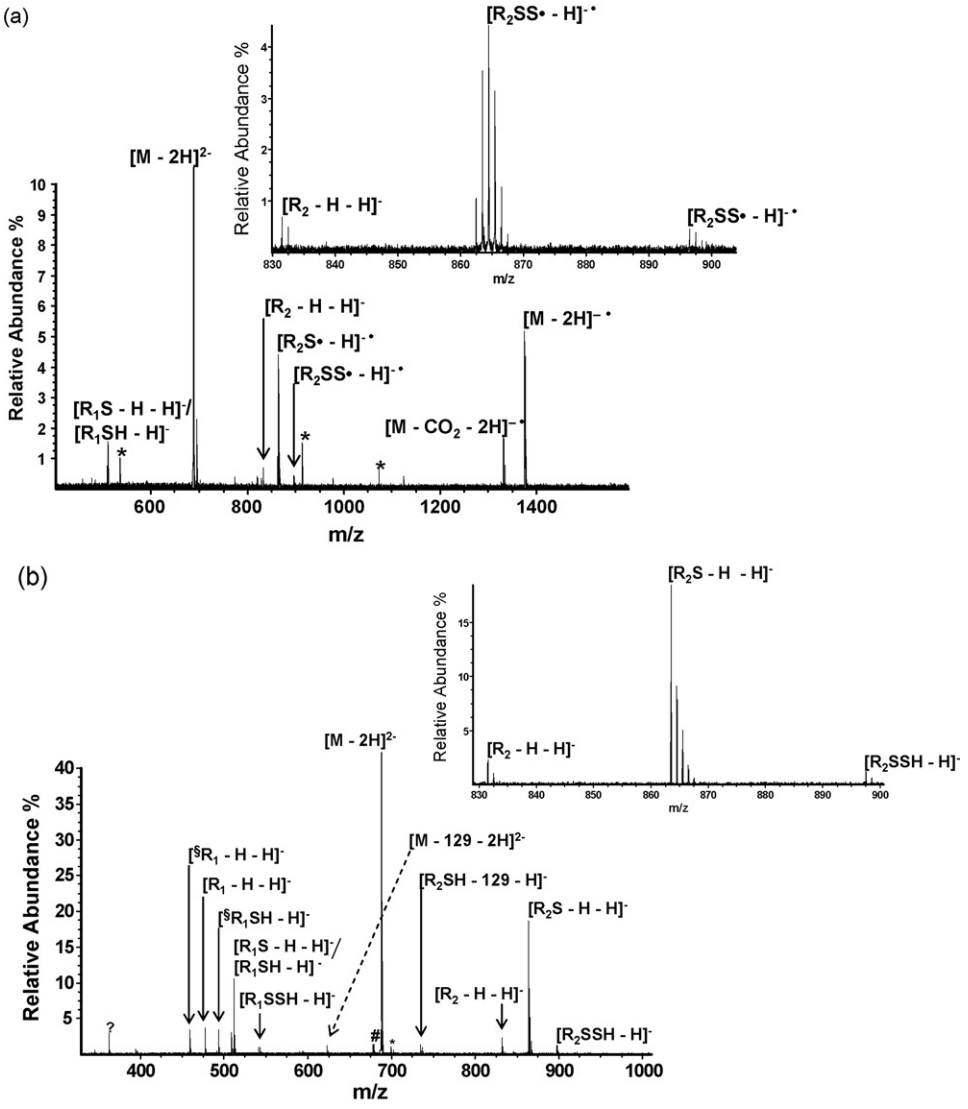


Fig. 3. EDD (a) and IRMPD (b) of the peptide pair C20A&C19B. In EDD, the charge reduced species dominates the spectrum, however, product ions corresponding to cleavage of S—S and C—S bonds in both peptide chains are also observed, similar to IRMPD. The product ion corresponding to chain R_1 is a mixture of two species. (*) noise peaks, (#) ammonia loss, and (§) water loss.

displays a higher relative abundance as compared to the peptides lacking an intramolecular disulfide.

The cleavages observed in EDD of the peptide pair C7A&C7B–C6A&C11A (Fig. 4a) cannot be readily explained within the previously proposed EDD mechanism [44] because there are no acidic sites close to the intermolecular disulfide bond. The glutamic acid at the fourth position in the R_1 peptide chain could possibly explain the cleavage of the intramolecular disulfide bond. However, it does not appear to be involved in cleavage of the intermolecular disulfide because the corresponding product ion containing the R_2 peptide chain is predominantly an even-electron species (see inset) whereas the product containing the R_1 peptide chain is observed both as a predominantly even-electron species (doubly charged) and as a predominantly radical species (singly charged). As mentioned above (and further discussed below), an alternative explanation could be direct electron detachment from the disulfide bond. IRMPD of the same species resulted in richer fragmentation than EDD (see Fig. 4b) in which both S–S (to form both $[RSH - nH]^{n-}$ and $[RS - nH]^{n-}$ ions ($n = 1, 2$), as above), C–S, and one backbone bond are cleaved. In addition, cleavage of the intramolecular disulfide bond is observed, as evidenced by the detection of the product ions, $[R_1 - 3H - 2H]^{2-}$, $[R_1 - S - 3H - 2H]^{2-}$, $[R_1 - 2S - 3H - 2H]^{2-}$. The former product ion corresponds to cleavage of the intramolecular S–S bond, while the two latter correspond to cleavage of the neighboring C–S bonds. One backbone product ion corresponding to a $[b_{12} + H_2O]$ ion of chain R_2 is observed. As discussed above, such product ions can be formed from elimination of the C-terminal glutamic acid residue in a rearrangement reaction [57–59].

3.2.2. C62&C68–C74&C96

A second example of EDD of a peptide pair containing both an intermolecular and an intramolecular disulfide bond, C62&C68–C74&C96 (see Scheme 1c), is shown in Fig. 5a. For this species, very limited fragmentation is observed. Neither

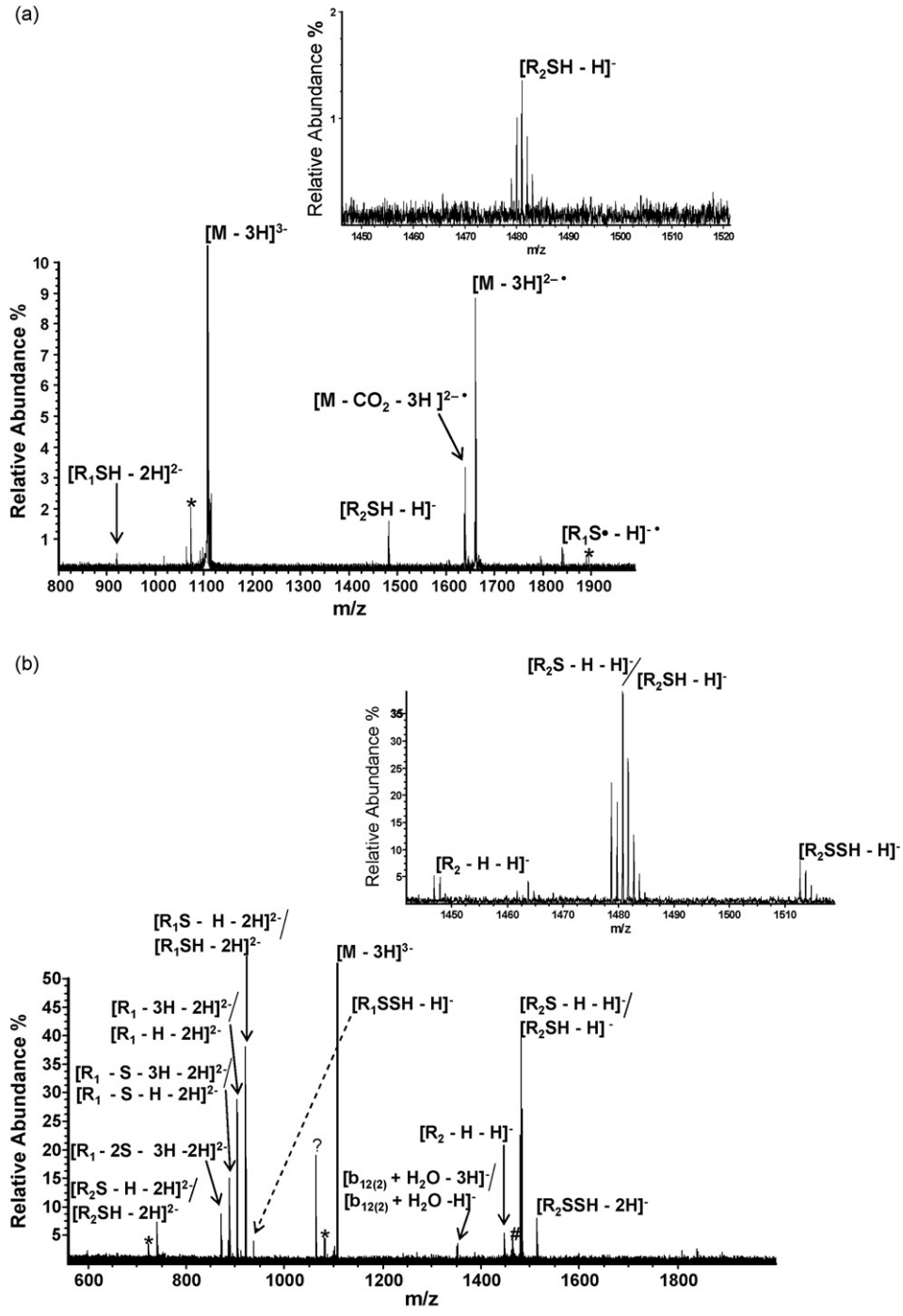


Fig. 4. EDD (a) and IRMPD (b) of the peptide pair C7A&C7B–C6A&C11A. No product ions corresponding to cleavage of C—S bonds are observed in EDD whereas both C—S and S—S bond cleavage is seen in IRMPD. The product ions $[R_1–3H–2H]^{2-}$, $[R_1–S–3H–2H]^{2-}$ and $[R_1–2S–3H–2H]^{2-}$ result from intramolecular bond cleavage in the R_1 peptide chain.

S–S, nor C–S bond cleavage is seen although charge reduction to form $[M-3H]^{2-\bullet}$ as well as CO_2 loss are observed. Because the two latter product ions were significantly more abundant than products corresponding to S–S bond cleavage for the C7A&C7B–C6A&C11A peptide pair discussed above (Fig. 4a), the absence of such products for the current peptide could be due to limited dynamic range. An alternative explanation for the low fragmentation efficiency of both C7A&C7B–C6A&C11A and C62&C68–C74&C96 could be that a different charge state (3^-) was fragmented as compared to the peptides with solely an intermolecular disulfide bond (2^-). These charge states were chosen for fragmentation because they constituted the most abundant species following electrospray ionization. The charge state effect in EDD has not been well established although one can envision that an increased precursor ion charge may lower the fragmentation efficiency due to increased Coulomb repulsion, which can reduce the electron interaction cross-section. However, in EDD of oligonucleotides, higher charge states (4^- and 3^-) were found

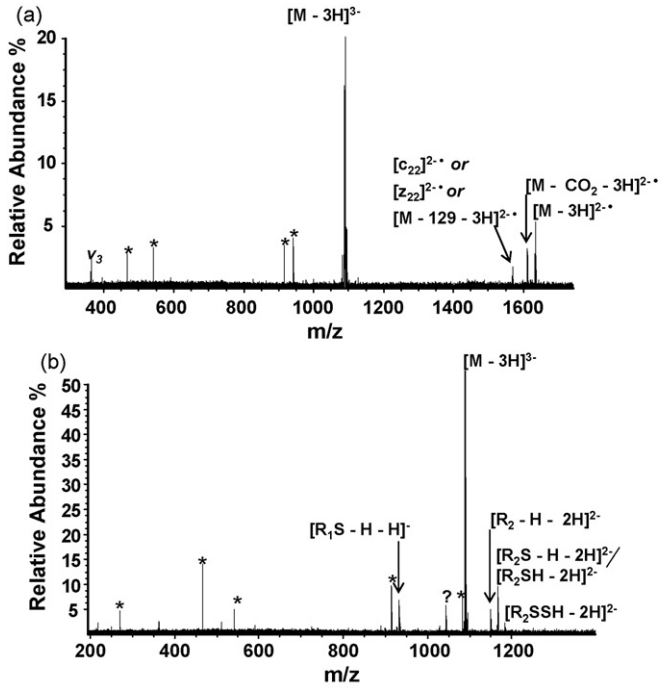


Fig. 5. EDD (a) and IRMPD (b) of the triply deprotonated ions of the peptide pair C62&C68–C74&C96. No product ions corresponding to S—S or C—S bond cleavage are observed in EDD although charge reduction, CO_2 loss, and neutral loss of 129 Da are detected. The latter product ion presumably corresponds to loss of the tryptophan side chain.

to dissociate more efficiently than doubly deprotonated ions. This behavior was attributed to a higher degree of gas-phase unfolding for the more highly charged ions (thereby preventing product ion pairs from remaining bound) [62]. For the peptides characterized here, gas-phase conformational flexibility is limited due to the presence of the intramolecular disulfide bond. Quadruply deprotonated ions were not observed, however, EDD of doubly deprotonated precursor ions resulted in a slightly decreased fragmentation efficiency (see supplementary data).

In addition to the $[M-3H]^{2-\bullet}$ and CO_2 loss product ions mentioned above, EDD of C62&C68–C74&C96 resulted in a product corresponding to neutral loss of 129. This species cannot result from elimination of a C-terminal glutamic acid residue because this peptide pair was produced by trypsin rather than Glu-C digestion. Possible assignments (within the 15 ppm criterion) are $c_{22(2)}{}^{2-\bullet}$ or $z_{22(2)}{}^{2-\bullet}$ from backbone cleavage within the R_2 peptide chain without S–S or C–S bond cleavage. However, because the latter cleavages dominated in all EDD spectra shown above and we did not previously observe any EDD product ions that resulted from backbone cleavage, we also considered alternative assignments. One possibility is neutral loss of C_9H_7N (129.058 amu), which corresponds to a tryptophan side chain. Loss of 129 was observed in CAD of fast atom bombardment-generated deprotonated tryptophan and has been proposed to result from charge migration (producing a deprotonated N-terminus) followed by formation of an indole anion and hydride transfer [63]. In ECD, neutral loss of 131.074 has been attributed to an even-electron fragment from the Trp side chain, originating from proton solvation onto Trp [64]. Consistent with the proposed EDD mechanism [44], loss of 129 in EDD could involve amide deprotonation, similar to CAD. Alternatively, deprotonation of the Trp side chain, followed by electron detachment from Trp may explain this behavior. However, it is also interesting to note that tryptophan has the lowest vertical ionization energy of all amino acids (7.07–11.61 eV), depending on conformation [65]. This IE can be lower than the ionization energy for the disulfide bond (8.46–9.1 eV) [56], thus suggesting that direct electron detachment from Trp can compete with detachment from disulfide bonds, as discussed in more detail below. On the other hand, loss of 129 was not observed in EDD of the peptide pair C30&C115 (Fig. 2a), which also contains a tryptophan residue. However, the C62&C68–C74&C96 peptide pair discussed here contains two Trp residues, which may strengthen this effect.

The IRMPD mass spectrum of the peptide pair C62&C68–C74&C96 is shown in Fig. 5b. This spectrum is less informative than the corresponding spectrum for the peptide pair C7A&C7B–C6A&C11A (Fig. 4b), which contained a mixture of S–S, C–S, and peptide backbone cleavages. However, it is more informative than the EDD spectrum for the same species (Fig. 5a) in that both S–S and C–S bond cleavage is observed although the former dominates.

3.3. The role of tryptophan in EDD

To further characterize the influence of tryptophan on the EDD behavior, we subjected a range of different Trp-containing peptides to electron irradiation. EDD of the doubly deprotonated peptide pEVNFSPGWGT-NH_2 (peptide 1) is shown in Fig. 6a. Here, selective cleavage on the N-terminal side of tryptophan is observed in the form of an abundant c_7 ion. In addition, neutral loss of 129 from the charge-reduced species is detected, as was observed for the peptide pair C62&C68–C74&C96 discussed above. The peptide H-GNLWATGHFM-NH_2 (peptide 2) displayed only limited fragmentation in EDD. However, an abundant product ion corresponding to loss of 129 Da and a $z_6{}^{\bullet}$ fragment ion corresponding to cleavage next to the tryptophan residue were observed (see supplementary data). By contrast, EDD of the peptide H-DYMGWMDF-NH_2 (peptide 3) did not result in loss of 129 (supplementary data). Here, the charge-reduced $[M-2H]^{-\bullet}$ species was the most prominent product ion followed by CO_2 loss. All these peptides contain one tryptophan residue, as does the peptide pair C30&C115. However, the latter peptide as well as peptide 3 did not display 129 loss. Those two peptides are different from the other two (peptides 1 and 2) in that two acidic sites are present (the two C-termina for C30&C115 and the two aspartic acid residues for peptide 3). Thus, amide or tryptophan side chain deprotonation is unlikely. However, peptides 1 and 2 only contain one acidic site (the C-terminus) and amide or tryptophan deprotonation is therefore much more likely for the doubly deprotonated precursors, consistent with the mechanism for tryptophan side chain loss proposed above.

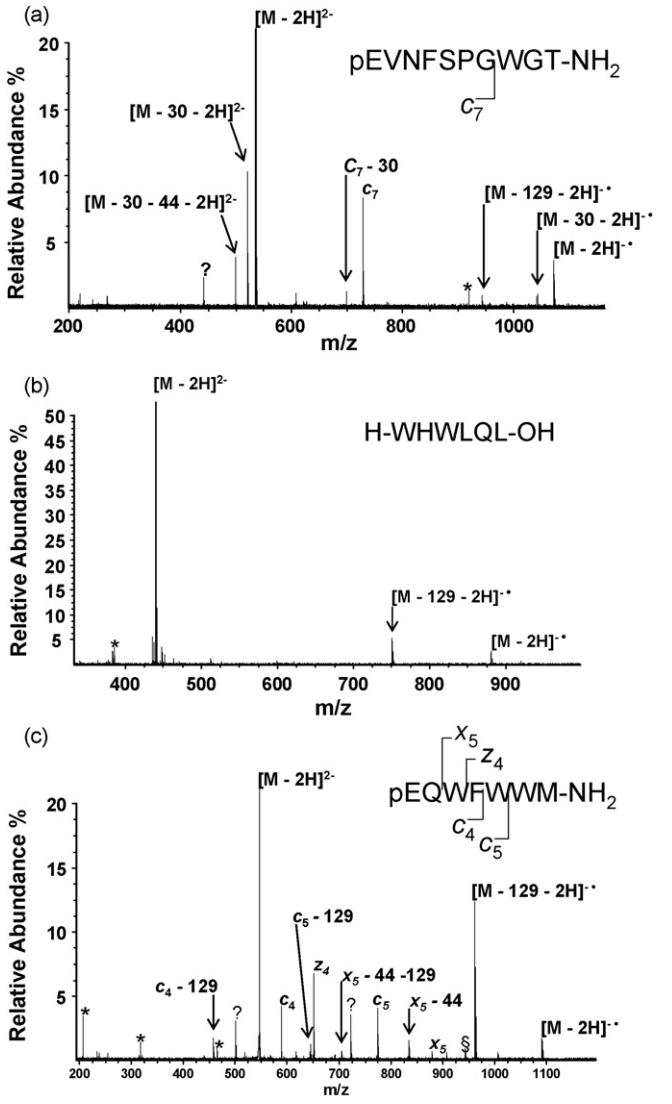


Fig. 6. EDD of tryptophan-containing peptides. Neutral loss of 129 (tryptophan side chain) is observed in all cases with an abundance correlating with the number of Trp residues. Peptide backbone cleavage next to tryptophan is also prevalent. In (a) neutral loss of 30 Da (HCHO, 30.0106 Da) corresponds to cleavage of a serine side chain while neutral loss of 44 Da (MeCHO, 44.0262 Da) corresponds to threonine side chain cleavage. In (c), neutral loss of 44 Da (•CONH2, 44.0136 Da) is observed from the x_5 product ion and attributed to the loss of the amidated C-terminus. Tryptophans in (c) are D-Trp. (*) noise peaks, (?) unidentified product ion, and (§) water loss.

Fig. 6b shows the EDD mass spectrum from the doubly deprotonated peptide H-WHWLQL-OH, which contains two Trp residues. Here, neutral loss of 129 constitutes the dominant product ion. Similarly, even more pronounced loss of 129 is detected in EDD of pEQWFWWM-NH_2, which contains three tryptophans (Fig. 6c). The latter spectrum also displays pronounced backbone cleavage although backbone product ions are less abundant than the product corresponding to neutral loss of 129. The two latter EDD spectra corroborate our claim that the presence of two Trp residues in C62&C68–C74&C96 can enhance the fragmentation pathway resulting in loss of 129 and thereby suppress disulfide cleavage.

3.4. EDD and IRMPD of intact insulin

EDD of quadruply deprotonated insulin (see supplementary data) resulted in formation of a charge reduced species and a product ion corresponding to loss of CO_2 from the charge reduced species. No product ions corresponding to cleavage of S–S or C–S bonds were observed. However, two disulfide bridges need to be cleaved in order to detect such products. Also, electron detachment from quadruply deprotonated species may be unfavorable due to increased Coulomb repulsion between ions and electrons, as previously discussed. Nevertheless, such behavior was observed in ECD of insulin cations [27]. Similarly, IRMPD of insulin anions (supplementary data) resulted in cleavage of both the intermolecular disulfide bonds to produce separated A and B chains. In addition, backbone cleavages of chain B were observed. These results are similar to those obtained from negative ion mode CAD of insulin [33]. In particular, one c-type product ion corresponding to cleavage N-terminal to cysteine was detected although this product can also be assigned as an internal fragment corresponding to the sequence LVEALYLVCGERGFFYTP of chain B minus water.

4. Conclusions

Electron detachment dissociation of multiply charged disulfide-bonded peptide anions results in preferential cleavage of C–S and S–S bonds in many cases. However, in the case of insulin for which multiple disulfide linkages are present, EDD did not provide any detectable disulfide bond cleavage. In addition, the presence of tryptophan in a polypeptide chain can alter the preferred fragmentation pathway in EDD and yield abundant product ions corresponding to the loss of its side chain. This behavior correlates with the vertical ionization energies of a disulfide bond and tryptophan, respectively. By contrast, the IRMPD fragmentation behavior was more consistent, showing strongly favored cleavage of both S–S and C–S bonds in all peptides investigated, even in cases where multiple disulfide linkages were present. Thus, the mechanism of disulfide bond cleavage appears to be different in IRMPD compared to EDD, further corroborated by the formation of mainly odd-electron species in EDD and exclusively even-electron species in IRMPD. Nevertheless, both these fragmentation techniques can be useful for probing disulfide bonding in peptide anions.

Acknowledgment

This work was supported by an Analytical Chemistry award from Eli Lilly & Company, a Dow Corning Assistant Professorship, and the University of Michigan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijms.2007.01.001.

References

[1] F.W. McLafferty, Tandem Mass Spectrometry, Wiley, New York, 1983.
[2] D.F. Hunt, J.R. Yates, J. Shabanowitz, S. Winston, C.R. Hauer, Proc. Natl. Acad. Sci. U.S.A. 83 (1986) 6233.
[3] K. Biemann, I.A. Papayannopoulos, Acc. Chem. Res. 27 (1994) 370.
[4] M.L. Gross, Acc. Chem. Res. 27 (1994) 361.
[5] G. Siuzdak, Proc. Natl. Acad. Sci. U.S.A. 91 (1994) 11290.
[6] J.R. Yates, J. Mass Spectrom. 33 (1998) 1.
[7] M. Mann, R.C. Hendrickson, A. Pandey, Annu. Rev. Biochem. 70 (2001) 437.
[8] R.N. Hayes, M.L. Gross, Meth. Enzymol. 193 (1990) 237.
[9] S.A. McLuckey, J. Am. Soc. Mass Spectrom. 3 (1992) 599.
[10] R.L. Woodin, D.S. Bomse, J.L. Beauchamp, J. Am. Chem. Soc. 100 (1978) 3248.
[11] D.P. Little, J.P. Speir, M.W. Senko, P.B. Oconnor, F.W. McLafferty, Anal. Chem. 66 (1994) 2809.
[12] R.B. Cole, S. Lemeillour, J.C. Tabet, Anal. Chem. 64 (1992) 365.
[13] M.D.A. Mabud, M.J. Dekrey, R.G. Cooks, Int. J. Mass Spectrom. Ion Process. 67 (1985) 285.
[14] A.L. McCormack, J.L. Jones, V.H. Wysocki, J. Am. Soc. Mass Spectrom. 3 (1992) 859.
[15] E.R. Williams, J.J.P. Furlong, F.W. McLafferty, J. Am. Soc. Mass Spectrom. 1 (1990) 288.
[16] M.S. Thompson, W.D. Cui, J.P. Reilly, Angew. Chem. Int. Ed. 43 (2004) 4791.
[17] R.A. Zubarev, N.L. Kelleher, F.W. McLafferty, J. Am. Chem. Soc. 120 (1998) 3265.
[18] B.A. Budnik, K.F. Haselmann, R.A. Zubarev, Chem. Phys. Lett. 342 (2001) 299.
[19] J.E.P. Syka, J.J. Coon, M.J. Schroeder, J. Shabanowitz, D.F. Hunt, Proc. Natl. Acad. Sci. U.S.A. 101 (2004) 9528.
[20] T.E. Creighton, Proteins Structures and Molecular Properties, W.H. Freeman and Company, New York, 1993.
[21] A. Fersht, Structure Mechanism in Protein Science, W.H. Freeman and Company, New York, 1999.
[22] C.N. Pace, G.R. Grimsley, J.A. Thomson, B.J. Barnett, J. Biol. Chem. 263 (1988) 11820.
[23] W.J. Wedemeyer, E. Welker, M. Narayan, H.A. Scheraga, Biochemistry 39 (2000) 4207.
[24] J.J. Gorman, T.P. Wallis, J.J. Pitt, Mass Spectrom. Rev. 21 (2002) 183.
[25] M.F. Bean, S.A. Carr, Anal. Biochem. 201 (1992) 216.
[26] M.D. Jones, S.D. Patterson, H.S. Lu, Anal. Chem. 70 (1998) 136.
[27] R.A. Zubarev, N.A. Kruger, E.K. Fridriksson, M.A. Lewis, D.M. Horn, B.K. Carpenter, F.W. McLafferty, J. Am. Chem. Soc. 121 (1999) 2857.
[28] T. Kocher, A. Engstrom, R.A. Zubarev, Anal. Chem. 77 (2005) 172.
[29] S.D. Patterson, V. Katta, Anal. Chem. 66 (1994) 3727.
[30] J.J. Gorman, B.L. Ferguson, T.B. Nguyen, Rapid Commun. Mass Spectrom. 10 (1996) 529.
[31] Y.M.E. Fung, F. Kjeldsen, O.A. Silivra, T.W.D. Chan, R.A. Zubarev, Angew. Chem. Int. Ed. 44 (2005) 6399.
[32] P.A. Chrisman, S.J. Pitteri, J.M. Hogan, S.A. McLuckey, J. Am. Soc. Mass Spectrom. 16 (2005) 1020.
[33] P.A. Chrisman, S.A. McLuckey, J. Proteome Res. 1 (2002) 549.
[34] M. Zhang, I.A. Kaltashov, Anal. Chem. 78 (2006) 4820.
[35] J. Zhou, W. Ens, N. Poppeschriemer, K.G. Standing, J.B. Westmore, Int. J. Mass Spectrom. Ion Process. 126 (1993) 115.
[36] N.P. Ewing, C.J. Cassady, J. Am. Soc. Mass Spectrom. 12 (2001) 105.
[37] K. Janek, H. Wenschuh, M. Bienert, E. Krause, Rapid Commun. Mass Spectrom. 15 (2001) 1593.
[38] T. Yagami, K. Kitagawa, S. Futaki, Rapid Commun. Mass Spectrom. 9 (1995) 1335.
[39] P.M. Bigwarfe, T.D. Wood, Int. J. Mass Spectrom. 234 (2004) 185.
[40] J. Jai-Nhuknan, C.J. Cassady, Anal. Chem. 70 (1998) 5122.
[41] J.H. Bowie, C.S. Brinkworth, S. Dua, Mass Spectrom. Rev. 21 (2002) 87.
[42] D. Bilusich, V.M. Maselli, C.S. Brinkworth, T. Samguina, A.T. Lebedev, J.H. Bowie, Rapid Commun. Mass Spectrom. 19 (2005) 3063.
[43] K.F. Haselmann, B.A. Budnik, F. Kjeldsen, M.L. Nielsen, J.V. Olsen, R.A. Zubarev, Eur. J. Mass Spectrom. 8 (2002) 117.
[44] F. Kjeldsen, O.A. Silivra, I.A. Ivonin, K.F. Haselmann, M. Gorshkov, R.A. Zubarev, Chem. Eur. J. 11 (2005) 1803.
[45] H.K. Kweon, K. Hakansson, Proceedings of the 53rd ASMS Proceedings of the 53rd ASMS Conference on Mass Spectrometry and Allied Topics, San Antonio, TX, June 5–9, 2005.
[46] J. Yang, J.J. Mo, J.T. Adamson, K. Hakansson, Anal. Chem. 77 (2005) 1876.
[47] D. Bilusich, C.S. Brinkworth, J.H. Bowie, Rapid Commun. Mass Spectrom. 18 (2004) 544.
[48] M.A. McFarland, A.G. Marshall, C.L. Hendrickson, C.L. Nilsson, P. Fredman, J.E. Mansson, J. Am. Soc. Mass Spectrom. 16 (2005) 752.
[49] K. Hakansson, H.J. Cooper, R.R. Hudgins, C.L. Nilsson, Curr. Org. Chem. 7 (2003) 1503.
[50] M.W. Senko, J.D. Canterbury, S.H. Guan, A.G. Marshall, Rapid Commun. Mass Spectrom. 10 (1996) 1839.
[51] P. Jolles, Angew. Chem. Int. Ed. 8 (1969) 227.
[52] L.F. Smith, Am. J. Med. 40 (1966) 662.
[53] A.P. Ryle, F. Sanger, L.F. Smith, R. Kitai, Biochem. J. 60 (1955) 541.
[54] B.A. Budnik, K.F. Haselmann, Y.N. Elkin, V.I. Gorbach, R.A. Zubarev, Anal. Chem. 75 (2003) 5994.
[55] D.T. Leeck, H.I. Kenttamaa, Org. Mass Spectrom. 29 (1994) 106.
[56] J.J. Butler, T. Baer, S.A. Evans, J. Am. Chem. Soc. 105 (1983) 3451.
[57] G.C. Thorne, S.J. Gaskell, Rapid Commun. Mass Spectrom. 3 (1989) 217.
[58] Z. Li, T. Yalcin, C.J. Cassady, J. Mass Spectrom. 41 (2006) 939.
[59] E.M. Marzluff, S. Campbell, M.T. Rodgers, J.L. Beauchamp, J. Am. Chem. Soc. 116 (1994) 7787.
[60] J. Mo, K. Hakansson, Anal. Bioanal. Chem. (2006).
[61] D.M. Horn, Y. Ge, F.W. McLafferty, Anal. Chem. 72 (2000) 4778.
[62] J. Yang, K. Hakansson, Proceedings of the 53rd ASMS Conference on Mass Spectrometry and Allied Topics, San Antonio, TX, 2005.
[63] R.J. Waugh, J.H. Bowie, R.N. Hayes, Int. J. Mass Spectrom. Ion Process. 107 (1991) 333.
[64] K.F. Haselmann, B.A. Budnik, F. Kjeldsen, N.C. Polfer, R.A. Zubarev, Eur. J. Mass Spectrom. 8 (2002) 461.
[65] D. Dehareng, G. Dive, Int. J. Mol. Sci. 5 (2004) 301.