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Disulfide bond cleavages observed in SORI-CID of three nonapeptides complexed with divalent transition-metal cations

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Tandem MS sequencing of peptides that contain a disulfide bond is often hampered when using a slow heating technique. We show that complexation of a transition-metal ion with a disulfide-bridge-containing nonapeptide yields very rich tandem mass spectra, including fragments that involve the cleavage of the disulfide bond up to 56% of the total product ion intensity. On the contrary, MS/MS of the corresponding protonated nonapeptides results predominantly in fragments from the region that is not involved in the disulfide bond. Eleven different combinations of three nonapeptides and three metal ions were measured using Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) combined with sustained off-resonance irradiation collision induced dissociation (SORI-CID). All observed fragments are discussed with respect to four different types of product ions: neutral losses, b/y-fragmentation with and without the disulfide bond cleavage, and losses of internal amino acids without rupture of the disulfide bridge. Furthermore, it is shown that the observed complementary fragment pairs obtained from peptide–metal complexes can be used to determine the region of the binding site of the metal ion. This approach offers an efficient way to cleave disulfide-bridged structures using low energy MS/MS, which leads to increased sequence coverage and more confidence in peptide or protein assignments. Copyright © 2007 John Wiley & Sons, Ltd.

KEYWORDS: peptides; disulfide bonds; transition-metal cations; FTICR-MS; SORI-CID

INTRODUCTION

Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) offers a collection of techniques for identification and structural investigation of a large variety of biomolecules in the gas phase.[1] Such studies require information obtained from both high mass accuracy measurements and tandem mass spectrometry (MS^n) strategies.[2] The high mass accuracy and sensitivity of FTICR-MS enable peptide identifications with high confidence in the analysis of complex peptide mixtures in a typical proteomics setup. Moreover, most tandem MS techniques currently used for peptide fragmentation are compatible with FTICR mass spectrometers. The amount and quality of information obtained from these MS^n spectra are strongly related with the identity, relative abundance and the number of product ions. In this paper, we report on unexpected fragmentation pathways of three similar nonapeptides using a so-called *slow heating fragmentation technique*.[3] The most unexpected fragments observed in these rich tandem mass spectra result from different cleavages of a disulfide bridge between the two cysteines. It will be shown that complexation of these peptides with transition-metal ions results in enhancement of this effect.

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Slow heating techniques for tandem MS

Tandem MS^n is an indispensable tool to structurally characterize a selected precursor ion by analyzing its dissociation products. The most common method for ion fragmentation is collision induced dissociation (CID), often used for sequencing tryptic peptides obtained in a proteomics experiment. However, for sequencing peptides and proteins with higher molecular masses (>2 kDa), CID is not an ideal method. For these purposes, slow heating techniques can be advantageous. With these techniques, the internal energy of a molecule is slowly increased to initiate molecular fragmentation. FTICR mass spectrometers are extremely suitable for the application of slow heating techniques such as infrared multiphoton dissociation (IRMPD),[4] blackbody infrared dissociation (BIRD),[5,6] ultraviolet photon dissociation (UVPD),[7–10] surface induced dissociation (SID),[11–14] on-resonance collision induced dissociation[15] or sustained off-resonance irradiation collision induced dissociation (SORI-CID).[16–18] These techniques are referred to as 'slow heating' because the trapped gas-phase ions are

collided with relatively low kinetic energies to induce ion activation and fragmentation. In SID, the internal energy is increased through collision of the ion of interest with a static target,[12–14,19,20] whereas in on-resonance CID and SORI-CID the collision target is mobile, i.e. neutral atoms of an inert gas like Ar or He are pulsed into the ICR cell. In SORI-CID the selected precursor is periodically excited and de-excited by applying, in the presence of a collision gas, a low-amplitude radio frequency pulse slightly off-resonance (−1500 Hz) compared to the cyclotron frequency of the selected ion.[21] During this process, the ions undergo multiple collisions with the neutral atoms of the collision gas. In each collision event, a part of the kinetic energy of the ion is converted into internal energy. In this way, the internal energy of the excited ions can be modulated, thereby enabling the study of the lowest energy fragmentation pathways. Compared with on-resonance excitation, the collision energy in SORI-CID is much lower because of the smaller kinetic energy of the ions. Another advantage over CID is that in SORI-CID the fragments are produced close to the axis of the cell, allowing a more efficient detection of the dissociation products. With IRMPD, BIRD and UVPD the trapped ions are dissociated by irradiation with an infrared (IRMPD) or an ultraviolet (UVPD) laser beam or they are activated through absorption of infrared photons (BIRD) from a heated ICR cell. Similar to the SORI experiment, the energy after multiple photon absorption is redistributed through intramolecular vibrations. The dissociation essentially follows the same type of fragmentation pathways as when using collision based dissociation techniques.

Disulfide bond cleavages in mass spectrometry

It is well known that disulfide bonds play an important role in defining and stabilizing the secondary and tertiary structure of folded proteins.[22] Many biologically relevant peptides contain one or more intramolecular disulfide bonds between different cysteine residues. Tandem MS sequencing of peptides that contain disulfide bonds is often hampered when using slow heating techniques. For example, the CID of multiply protonated insulin with two intramolecular linkages results in incomplete sequence coverage.[23] In general, sequencing of multiply protonated peptides with intramolecular disulfide bonds is limited to backbone fragments that do not contain the disulfide linkage. This reduces the amount of information that can be extracted using such MS^n techniques. A common approach to overcome this problem is to reduce and alkylate the disulfide bonds prior to MS^n analysis. This preparation step is time consuming and, therefore, not desirable for high-throughput proteomics applications. Moreover, the site-specific information on the exact location of the S–S bond linkages is lost following reduction. Another approach of analyzing cysteine-bridged peptides is using high-energy CID,[24,25] MALDI in-source decay[26,27] or post-source decay,[26–29] or MALDI combined with ion trap MS.[30] In addition, ion trap MS has been used both in the positive[23] and negative[31] modes to study peptides/proteins that contain disulfide linkages. A powerful method for dissociation of multiply charged proteins that contain disulfide bridges is electron capture induced dissociation (ECD) introduced by McLafferty and coworkers.[32,33] It has been shown that ECD has the potential for cleaving and mapping disulfide bonds present in peptides and proteins.[34,35] Until now, ECD is implemented only on FTICR instruments, whereas compatibility with three-dimensional quadrupole ion traps would make it a more accessible fragmentation technique.[36,37] ECD of peptides requires multiple protonation, which can be difficult to be achieve. To overcome this hurdle, metal ions can be used as charge carriers in peptides. Moreover, the presence of a certain metal ion may be pivotal for the biologic activity of the peptide. Often the secondary and/or tertiary structure of a peptide (or protein) is changed by a co-factor such as a metal ion or a small nucleotide. A well-known example is the hormone oxytocin (OT), for which the presence of a transition-metal cation such as Cu or Zn is essential for binding to its cellular receptor. The ECD efficiency does not increase by using a metal ion; however, it has been shown that metalated peptides provide complementary ECD fragments compared to protonated species.[38,39] In line with this is a recent study on substance P, in which complexation with divalent metals resulted in very rich ECD spectra of triply charged species.[38,39] Structural investigation of OT complexed with different divalent transition-metal ions showed remarkable differences in the ECD spectra.[40] Here, the observed specific fragmentation pathways for different charge carriers possibly result from structural differences between the OT–metal ion complexes. For OT complexed with Ni^{2+}, Co^{2+} or Zn^{2+} the most abundant ECD fragments included the cleavage of the disulfide bond. However, the $[OT + Cu^{2+}]$ complex showed mainly b-type ions that did not result from the cleavage of the disulfide bond. In the latter case 15% of the fragments included a disulfide bond cleavage, whereas for other OT–metal ion complexes 60–70% of the fragments included one cysteine residue only. This atypical ECD behavior of the $[OT + Cu^{2+}]$ complex inspired us to investigate this compound with a slow heating technique such as SORI-CID in more detail.

Rationale of this work

In this work, the SORI-CID fragmentation pathways of three different nonapeptides containing one disulfide bond are studied. The effect of complexation with different divalent transition-metal cations compared with protonated peptide will be discussed in terms of cleavage efficiency of the disulfide bond. Further, it will be investigated whether the differences between the measured tandem mass spectra can be correlated to possible conformational changes in the peptide due to the presence of a metal ion.

OT (CysTyrIleGlnAsnCysProLeuGly) was chosen as a model compound for these studies on the basis of the remarkable results from earlier work.[40] Two peptide analogs were chosen to explore possible structural correlations or differences, namely, vasopressin (VS, CysTyrPheGlnAsnCysProArgGly) and Thr4-Gly7-OT (TGOT, CysTyrIleThrAsnCysGlyLeuGly). All three peptides contain a disulfide bridge between the cysteines at positions 1 and 6 and are amidated at the *C*-terminus. Usually, for

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OT the peptide region that is closed by the disulfide bond is called the *tocin ring* and the remaining part is referred to as the *tail*. The tocin ring is a rather rigid structure, whereas the tail is flexible. In this paper, the terms 'tocin ring' and 'tail' will also be used for describing regions of the other two nonapeptides owing to the structural similarities with OT. Both OT and VS exert various hormonal effects. OT is responsible to elicit contraction of uterus smooth muscle at term and of myoepithelial cells that surround the alveoli of the mammary gland during lactation.[41] OT also plays a decisive role in the affiliation process in mammals, such as maternal behavior and infant separation distress.[42] VS facilitates water re-absorption by the kidney and the contraction of smooth muscle cells in arteries.[41,43] Moreover, more recent studies showed that these peptides are involved in numerous cognitive processes such as memory and learning.[41] OT and VS are biologically activated by essential elements such as metal ions (Zn^{2+}, Ni^{2+} or Co^{2+}). The presence of the divalent metal ion could drastically change the peptide conformation with implications in the binding process of these peptides to their receptors. The role of these metal ions for the interaction process of OT with its receptor has been highlighted earlier.[44,45] It has been shown that binding of OT to its receptor is enhanced in the increasing order of Zn^{2+}, Ni^{2+} and Co^{2+} but is negligible[43] in the presence of Cu^{2+}. However, it has not been elucidated whether the metal ion interacts first with the peptide or the receptor, or with both.

EXPERIMENTAL

Mass spectrometry

All the experiments were performed using a heavily modified Bruker APEX 7.0eT FTICR mass spectrometer equipped with a 7 T superconducting magnet and an infinity cell. The ions generated by an electrospray ion source are accumulated (300–500 ms) in an octopole ion trap[46] prior to being transferred to the ICR cell via two quadrupole ion guides. Argon was used for gas-assisted dynamic trapping of the ions. High-resolution mass spectra revealed that all the nonapeptides complexed with divalent metal cations resulted in precursor ions of the type $[peptide + metal]^{2+}$. The full isotopic cluster of a doubly charged precursor ion was isolated using a stored waveform inverse Fourier transformed (SWIFT) excitation pulse.[47] In some cases, an additional SWIFT-isolation was applied to avoid noise signals at the lower mass range. Fragmentation of protonated and divalent-metal-containing peptides was carried out using SORI-CID.[16] The isolated ion clusters were excited kinetically using a SORI pulse with 250 cycles at a frequency −1500 Hz away from the cyclotron frequency of the isolated ion. For fragmentation, argon collision gas was pulsed into the cell at a pressure of $\sim 10^{-7}$ mbar. During detection, the pressure in the ICR cell was less than 10^{-9} mbar. All experimental parameters were controlled using software and hardware developed in-house as part of the continual evolution of this proteomics/fundamental studies instrument.[48,49] Data analysis was carried out both manually and automatically using in-house developed processing tools.[50]

Sample preparation

All three nonapeptides, namely, OT, TGOT and Arg8vasopressin, were purchased from Sigma Aldrich and used without further purification. All three peptides contain a disulfide bond between Cys-1 and Cys-6 and are amidated at the *C*-terminus. Sample solutions of 49 : 49 : 2 water : methanol : acetic acid at a concentration of 20 μM were prepared. Freshly prepared complexes of the peptides with Ni^{2+}, Zn^{2+} and Cu^{2+} were obtained using the corresponding acetate salt of the metal ion in 25 times excess in the absence of acetic acid. Electrospray ionization was carried out using New Objective Picotips at 500 nl/min and an electric potential difference of approximately 2100 V.

RESULTS AND DISCUSSION

Nomenclature

The assignment and designation of peptide fragments are based on Roepstorff–Fohlman–Biemann nomenclature.[51–53] All observed backbone cleavages in the three studied nonapeptides are typical b or y, in which the charge is retained at the *N*- or the *C*-terminus, respectively. For calculation of the exact masses of the fragments for each cysteine residue, one hydrogen atom is subtracted because of the disulfide bridge. Backbone fragments that result from cleavage between residues 1 up to 6 ('tocin ring') require additional cleavage of the disulfide bridge. Basically, this disulfide bridge can be cleaved at three different positions. The 'regular' fragment ion results from S–S cleavage. Two other types are referred to as '+S or −S' when the backbone bond is cleaved in combination with an S–C bond. These fragment ions therefore contain either two or zero sulfur atoms. In principle, the fragment ions observed in the tandem mass spectra of nonapeptides complexed with Zn^{2+}, Ni^{2+} or Cu^{2+} retained the metal ion. It will be mentioned explicitly when this is not the case (Table 2). Finally, the internal fragments resulting from multipoint cleavages in the backbone of the precursor ion[54,55] or the dissociation products are denoted with the three-letter code of the amino acid that is lost.

Four different types of product ions in SORI-CID spectra

For each of the three nonapeptides, three different tandem MS spectra (SORI-CID after SWIFT-isolation of the doubly charged precursor ion) were obtained after complexation with Zn^{2+}, Ni^{2+} or Cu^{2+} ions. Similarly, MS/MS was carried out on doubly protonated OT and VS. In the case of TGOT the doubly protonated species was not observed in the mass spectrum, probably because of the absence of basic amino acids. Thus 11 SORI-CID spectra were obtained. These will be discussed according to the different types of product ions as depicted in Fig. 1. Each product ion results from a neutral loss, a typical b/y-fragmentation or a combination thereof. In addition, certain product ions require the cleavage of the disulfide bridge, which occurs either at S–S or at C–S. Basically, four main types of product ions are observed in the spectra. The first results from single or multiple neutral loss of NH_3, H_2O or CO from the precursor ion (I). The second follows 'regular' b/y-fragmentation of the peptide backbone

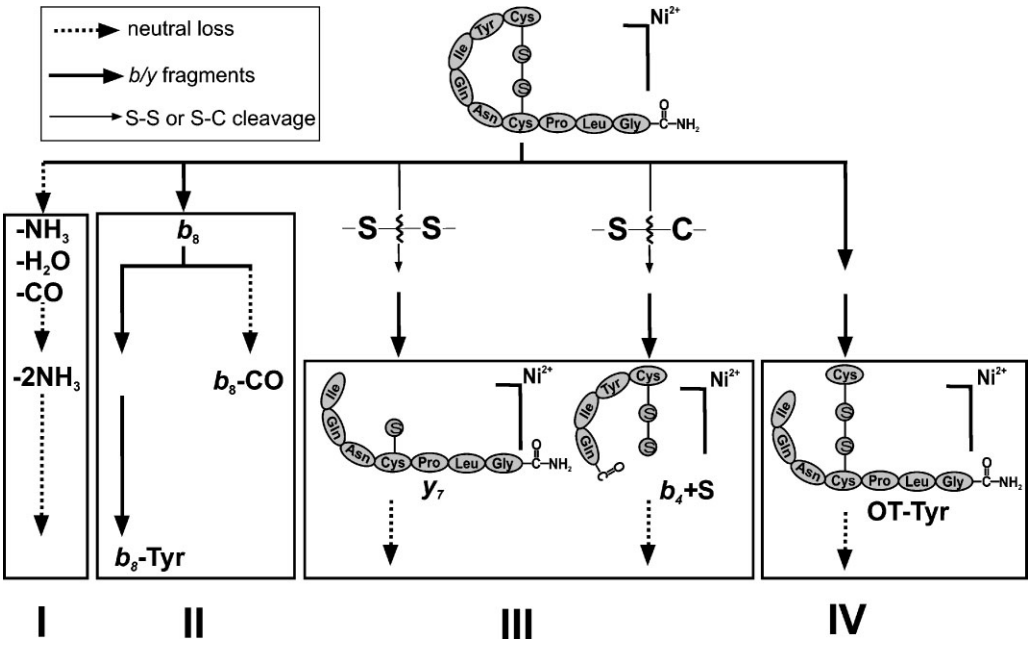

Figure 1. Overview of different types of product ions observed in SORI-CID spectra of protonated or metal-complexed nonapeptides. As an example, a few of the product ions observed upon SORI-CID of OT complexed with Ni^{2+} are shown. Note that the sequence of events is chosen arbitrarily, e.g. '$b_8 - CO$' may also result from the loss of CO followed by b/y-fragmentation. The ion 'OT − Tyr' may fragment further to '$b_8 - Tyr$' via b/y-fragmentation.

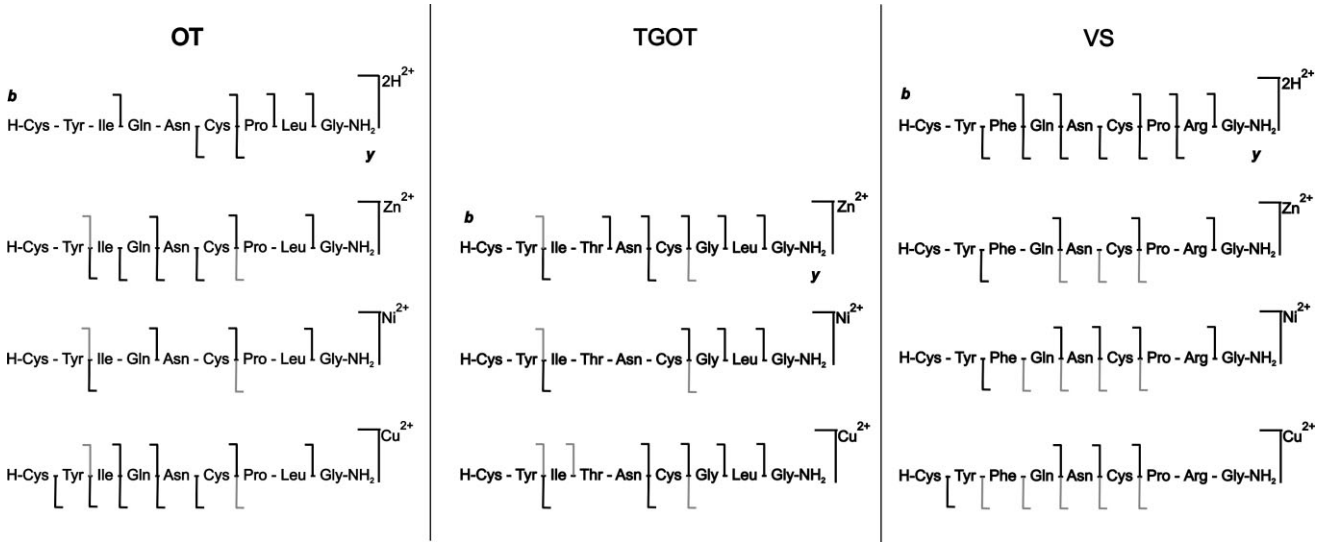

Figure 2. Summary of all fragments observed in 11 different SORI-CID spectra. All fragments displayed in black contain the metal cation as indicated with the precursor ion. Fragments displayed in grey do not contain the metal cation.

tail (II), resulting in observed product ions b_6, b_7, b_8, y_3 and y_2. The third type results from the b/y-fragmentation in the tocin ring, including a cleavage of the disulfide bridge (III). As was explained earlier (see Section 'Nomenclature'), this fragmentation pathway results in observed fragments such as b_4, $b_4 + S$, y_7 or $y_4 - H_2S$. These product ions are rather unexpected, since disulfide bonds usually are not affected by slow heating techniques. The fourth and last type of product ions here considered also requires multipoint cleavages (IV), as is the case for fragmentation of type III. Here, one or more internal amino acids are lost from the precursor through typical b/y-cleavages, whereas the disulfide bridge remains intact. Such losses are observed from both the tocin ring and the tail. Complimentarily, in most cases of multiple residue loss, these internal amino acids are observed in the spectrum (e.g. $[Tyr + Ile]^+$ or $[Tyr + Ile + Thr]^+$). The sequence of events to result in type I, II, III or IV ions is chosen arbitrarily in Fig. 1. The results from all 11 SORI-CID spectra are summarized in Fig. 2. In Table 1, all the identified product ions are categorized according to the four types of fragmentation pathways. The intensity of each product ion was determined from all corresponding peak intensities in the isotope cluster. In the case of a Ni^{2+}- or Cu^{2+}-complexed peptide, at least three isotopes were observed in one cluster, whereas in the case of Zn^{2+} at least four peaks were taken into account. Thus, the obtained intensities are shown relative to

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Table 1. Product ion intensities in SORI-CID spectra of three different nonapeptides

Peptide	Assigned fragments (no.)	Assigned fragments (%PII)	I (neutral loss)	II (tail b/y)	III (ring b/y)	IV (internal loss)
$[OT + 2H^+]$	41	99	23	64	1	12
$[OT + Zn^{2+}]$	25	60	10	45	37	8
$[OT + Ni^{2+}]$	20	86	44	45	8	3
$[OT + Cu^{2+}]$	55	87	16	20	50	14
$[TGOT + Zn^{2+}]$	36	58	19	24	56	1
$[TGOT + Ni^{2+}]$	26	75	51	34	14	1
$[TGOT + Cu^{2+}]$	34	76	7	30	51	12
$[VS + 2H^{2+}]$	40	93	16	61	1	22
$[VS + Zn^{2+}]$	16	80	12	47	40	1
$[VS + Ni^{2+}]$	17	79	9	48	43	0
$[VS + Cu^{2+}]$	28	52	26	21	53	0

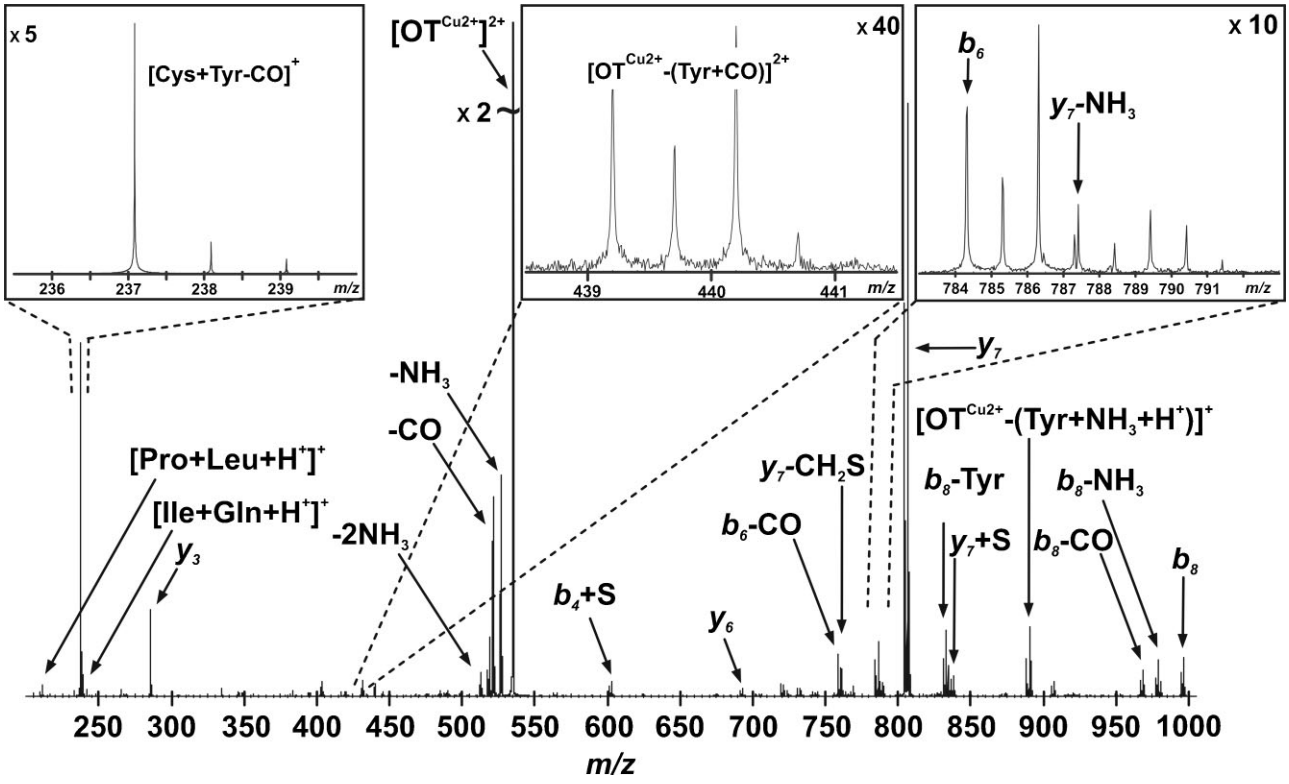

Figure 3. SORI-CID spectrum of $OT + Cu^{2+}$ obtained after SWIFT-isolation of the doubly charged Cu^{2+}-complexed nonapeptide. Note that all ions contain a Cu^{2+} ion, except $[Cys + Tyr - CO]^+$ and y_3.

the total intensity of all assigned product ions. The total number of assigned fragments in each MS/MS spectrum varied between 16 and 55. In most of the assignments, the mass accuracy was below 5 ppm. Mass accuracies between 5 and 15 ppm were usually due to significant intensity differences (more than 100-fold) between the fragments used for calibration and the ion of interest. Also shown in Table 1 are the assigned fragments as a percentage of the total product ion intensity (%PII). For most species, more than 75% of PII is assigned. For $VS + Cu^{2+}$, $OT + Zn^{2+}$ and $TGOT + Zn^{2+}$, a significant amount of PII was not assigned (48, 40 and 42%, respectively), mostly because of overlap with other isotope clusters in the mass spectrum (an example is shown in Fig. 3, see also Section 'Localization of the transition-metal ion in the peptide complex'). Moreover, it is evident that the typical isotope profiles of the transition metals add complexity to the spectrum. In the following sections, the SORI-CID results will be discussed with respect to the four types of product ions depicted in Fig. 1.

Small neutral losses from the precursor ion (I)

Single or multiple losses of small neutral species (NH_3, H_2O and CO) from the precursor ion are observed in all 11 spectra, however, with significant variation in relative abundances (type I product ions in Table 1). For OT and TGOT the highest total peak intensity of neutral losses is

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observed in the Ni^{2+}-complexed peptide and a low total peak intensity in the Cu^{2+} complex, whereas for VS the opposite is true. As is clear from Fig. 1, small neutral losses are also observed from product ions of type II, III or IV. In total, this additional loss accounts for 10–50% of the thus resulting ions, which remain categorized as b/y-fragments (type II, III or IV). Notably, in the MS/MS spectrum of $VS + Cu^{2+}$ the loss of H_2S is observed in different product ions [i.e. $M - H_2S$, $M - (H_2S + NH_3)$ and $M - (H_2S + NH_3 + H_2O)$]. This results from a rather unexpected cleavage of the disulfide bond, which will be discussed in more detail in Section 'Cleavage of the disulfide bridge (III)'.

'Regular' b/y-product ions from the peptide backbone tail (II)

Fragmentation of the backbone tail through b/y-cleavages accounts for 20% ($OT + Cu^{2+}$) to 64% ($OT + 2H^{2+}$) of PII (type II product ions in Table 1). The two protonated species (OT and VS) show the highest relative amount of tail fragments owing to the absence of type III ions [see also Section 'Cleavage of the disulfide bridge (III)']. The observed fragments in the peptide backbone tail are b_6, b_7, b_8, y_3 and y_2. Note that in none of the 11 different spectra did *all* of these ions occur at once. The complete series of *b*-ions from the tail are observed in all spectra from TGOT, which is complexed with a metal ion. Owing to the presence of proline in OT and VS, it is expected that b_6 and y_3 ions dominate the type II product ions. This effect is illustrated in Table 2, where the intensities of b_6 and y_3, and the sum of all remaining type II ions are depicted as the percentage of the total product ion intensity. As is clear from Table 2, this effect of proline is observed in $OT + Zn^{2+}$ and $OT + Ni^{2+}$. Remarkably, in both $OT + 2H^{2+}$ and $OT + Cu^{2+}$ the fragment ions b_7 and b_8 dominate or at least contribute significantly to the tail fragments. These findings can be explained as a result of different structural conformations of OT upon complexation with different metal cations. In contrast to OT, for all species of VS the ions b_6 and y_3 dominate the tail fragments. In the case of $VS + Zn^{2+}$ also, intense b_8-ions are observed. In all species of TGOT, the ions b_6 and y_3 appear in the SORI-CID spectra. These ions do not dominate the tail fragments, which is expected owing to the change of proline in glycine at position 7. For all metal-complexed nonapeptides, the y_3-fragment appears in the SORI-CID spectrum as a singly protonated ion. The only exception is $OT + Cu^{2+}$, in which both protonated and metal-containing y_3 are observed in the spectrum in the ratio of 7 : 1. From this observation of protonated y_3-ions, it is concluded that the metal cation is complexed with amino acids between 1 and 6. This is in agreement with the fact that in all spectra of metal-complexed nonapeptides the b_6-ion contains the doubly charged metal cation. The location of the metal cation in the peptide complex will be discussed in more detail in Section 'Localization of the transition-metal ion in the peptide complex'. Finally, in some spectra, an internal amino acid loss from a b_8-ion is observed ($OT + Cu^{2+}$, $OT + Ni^{2+}$, $OT + 2H^{+}$). One example is described in Fig. 1, i.e. $b_8 - Tyr$. It cannot be determined whether this product ion results from first b_8-formation and then loss of Tyr, or in the reverse order, or a combination of these two possibilities.

Table 2. Product ion intensities of b_6 and y_3 compared to other type II fragments. The numbers in this table indicate the percentage of PII

Peptide	b_6	y_3	All other type II ions (tail b/y)
$[OT + 2H^{+}]$	13	4	47
$[OT + Zn^{2+}]$	26	16	3
$[OT + Ni^{2+}]$	29	12	4
$[OT + Cu^{2+}]$	4	3	13
$[TGOT + Zn^{2+}]$	4	2	18
$[TGOT + Ni^{2+}]$	12	5	17
$[TGOT + Cu^{2+}]$	3	1	26
$[VS + 2H^{2+}]$	36	18	7
$[VS + Zn^{2+}]$	14	15	18
$[VS + Ni^{2+}]$	19	27	2
$[VS + Cu^{2+}]$	16	5	0

Cleavage of the disulfide bridge (III)

As is indicated in Fig. 1, product ions of type III require a two-step process. Both a b/y-fragmentation in the tocin ring and cleavage of the disulfide bridge are necessary for the formation of b_2, b_3, b_4, b_5, y_8, y_7, y_6, y_5 and y_4, and derivatives thereof (such as $b_4 + S$). From Table 1, it can be seen that SORI-CID fragmentation of the two protonated species hardly yields any ion of type III. This is in agreement with earlier studies, in which it was found that disulfide bridges hamper peptide sequencing using MS/MS methods (see also Section 'Disulfide bond cleavages in mass spectrometry'). Surprisingly, when complexed with Cu^{2+} or Zn^{2+}, all three nonapeptides yield significant amounts of product ions of type III (varying from 37 to 56% of PII). As a result, these SORI-CID spectra are richer than those of doubly protonated precursor ions and thus show greater peptide sequence coverage. In addition, the location of the disulfide bridge is reflected in the product ions. In the case of complexes with Ni^{2+}, type III ions are less abundant for OT and TGOT (8% and 14% respectively), whereas for $VS + Ni^{2+}$ 43% of PII results from cleavages of the disulfide bridge. This increase in cleavage efficiency of the disulfide bond is likely the result of conformational differences between the different metal-complexed peptides. Earlier, we also observed remarkable differences in the ECD spectra of metal-complexed OT depending on the type of metal ion used.[40] Furthermore, it is known that the conformation of OT changes upon complexation with different metal cations.[45] Also, the biological activity of OT depends strongly on the type of transition-metal cation,[44] which relates intuitively to the conformation of the metal-complexed peptide. Finally, the loss of H_2S is observed in metal-complexed peptides, both from the precursor ion $[VS + Cu^{2+}$, see also Section 'Small neutral losses from the precursor ion (I)'] and from b- or y-ions from the tocin ring (b_3, b_4, b_5, b_6, y_5, y_4).

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Loss of internal amino acids via b/y-fragmentation (IV)

Product ions of type IV result from sequential b- and y-ion formation. When this two-step process occurs in the tocin ring, both the 'lost' internal amino acid(s) and the remaining M (internal loss) can be expected in the MS/MS spectrum, provided that the disulfide bridge remains intact. Such ions are actually observed in five of the SORI-CID spectra here discussed and account for 8 to 14% of PII. In the case of protonated VS, the intensity is even 22%, mainly due to an abundant fragment ion pair $[M - (Phe + Gln + H^+)]^+/[Phe + Gln + H^+]^+$. Both single and multiple amino acid losses from the precursor are seen, where the latter process results in complementary product ions such as $[Tyr - Ile]^+$ or $[Tyr - Ile - Thr]^+$. These complementary fragments are detected as protonated species and never contain a metal cation. This fragmentation pathway is discussed in more detail in the next section. Furthermore, from Table 1 it can be seen that VS complexed with metal ions hardly yields any type IV ions upon SORI-CID. This fragmentation pathway also holds for Ni^{2+}-complexed OT and TGOT and TGOT + Zn^{2+}.

Localization of the transition-metal ion in the peptide complex

In all the discussed SORI-CID spectra of the nine metal-complexed species, multiple product ions are present that do not contain the metal cation. Only in a few cases, the absence of the metal ion is clear from the isotopic distribution of the fragment ion. When the absence of the metal ion cannot be determined unambiguously owing to the overlap between different clusters and low intensities of isotopes at lower masses, the assignments are based on mass accuracy. As an example, the SORI-CID spectrum of OT + Cu^{2+} is shown in Fig. 3. The isotope profile of the ion at m/z 237 is in good agreement with a distribution lacking Cu^{2+}, whereas at m/z 439 a clear Cu^{2+}-like isotope profile is observed. The magnification in Fig. 3 at m/z 784 shows an example of overlapping clusters in a SORI-CID spectrum (mentioned in Section 'Four different types of product ions in the SORI-CID spectra'). We hypothesize that fragments that lack the metal ion have a significant role in determining the location of the metal in the peptide complex in the gas phase. Moreover, the corresponding fragments that contain the metal ion corroborate these data. To test this idea, the OT + Zn^{2+} species is chosen because earlier work was carried out on the localization of the metal ion in this peptide complex.[44,45] Using molecular modeling it was found that the metal ion coordinates to amino acids in the tocin ring (Tyr-2, Ile-3, Gln-4 and Cys-6), and also may have interaction with amino acids from the tail (Pro-7 and Leu-8).[45] In Fig. 4, two strong fragment ion pairs can be seen, namely, b_2/y_7 and b_6/y_3. In the first pair, the Zn^{2+} remains on the y-ion, whereas in the second Zn^{2+} is present in the b-ion cluster. From these pairs, it follows that it preferably interacts with Ile-3, Gln-4, Asn-5 and/or Cys-6 in the tocin ring. Additional the y-fragments in this part of the peptide (y_4–y_6) are of low intensity, and therefore unambiguous localization of Zn^{2+} is not possible from these ions. Internal losses of amino acids are observed from fragment ions in the mass spectrum that do not contain Zn^{2+}. This suggests that these amino acids are not, or at least not preferentially, involved in the Zn^{2+} complexation in the tocin ring. This would leave Asn-5 and Cys-6 for interaction with Zn^{2+}; however, an intense b_4-ion containing the metal is observed. This b_4-ion actually is a 'b_4 + S' fragment, implying that Zn^{2+} remains on the b_4 part when the two sulfurs from the disulfide bridge are also included. Hence, all data point toward the presence of Zn^{2+} in the tocin ring. Additional fragments that result from this part of the peptide indicate interaction of the metal ion with Ile-3, Gln-4, Asn-5 and/or Cys-6. In all other metal-complexed nonapeptides, the typical fragment ion pair b_6/y_3 is highly abundant in the SORI-CID spectra, implying the presence of the metal ion in the tocin ring. The b_2/y_7 pair is present in all species of OT and TGOT; however, the b_2-ion is not present in the spectra of VS. In the case of OT + Cu^{2+} both protonated and metal-containing y_3 are observed in the spectrum [ratio 7 : 1, see also Section 'Regular' b/y-product ions from the peptide backbone tail (II)']. In addition, the b_6-ion is present both with and without the Cu^{2+} ion (ratio 11 : 1). This strongly indicates a mixture of two different species, i.e. one species with Cu^{2+} attached to the tail and the other with Cu^{2+} coordinating in the tocin ring.

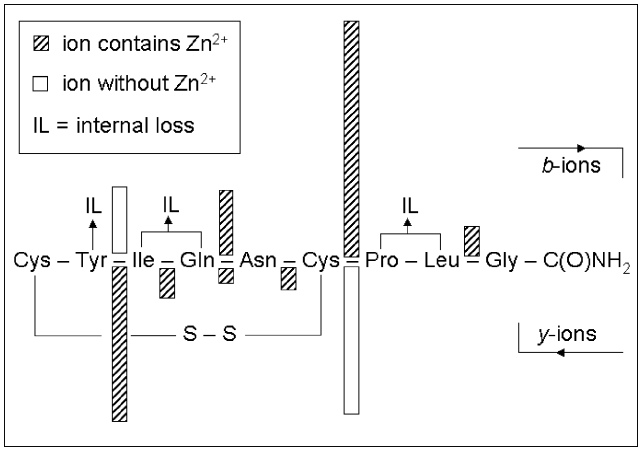

Figure 4. Overview of fragment ions obtained from SORI-CID of OT + Zn^{2+}. The length of the bars correlates linearly with the ion intensity.

CONCLUSIONS

It was shown that SORI-CID of a disulfide-bridge-containing nonapeptide (OT, TGOT or VS) complexed with a divalent transition-metal ion (Ni^{2+}, Cu^{2+} or Zn^{2+}) resulted in four types of dissociation products. First, small neutral mass losses from the precursor accounted for 7% up to 51% of the product ion intensity. Surprisingly, VS + Cu^{2+} showed the loss of H_2S as a neutral. Second, for all species, high intensities of backbone fragments resulting from cleavage of the tail region were observed (20–64% PII). For all metalated peptides, the b_6/y_3 ion pair suggested the presence of the metal ion in the tocin ring. Third, SORI-CID spectra of Zn^{2+}- or Cu^{2+}-complexed peptides showed abundant cleavages of the disulfide bond (37–56%). In the Ni^{2+}-complexed peptides of OT and TGOT, these ions are of lower intensities,

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whereas in the protonated species rupture of the disulfide bridge was observed only in trace amounts. This observed cleavage of the disulfide bridge upon complexation of a divalent transition-metal ion to a peptide is likely a result of induced conformational changes. Finally, in 5 out of 11 different species, single or multiple internal residue losses were observed (8–22% PII). It was shown that these internal losses in combination with fragment ion pairs could be used to localize of the complexation of the metal ion. The results presented here highlight the advantages of using transition-metal-cationized complexes for sequencing disulfide-bridged structures of peptides (and proteins) using slow heating techniques such as SORI-CID. This aspect is important because numerous biologically relevant peptides and proteins contain one or more disulfide bonds that often hamper MS/MS sequencing of amino acids in the bridged region.

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