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Structural studies on protein O-fucosylation by electron capture dissociation

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Dedicated to Professor Alan G. Marshall on the occasion of his 60th birthday.

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Abstract

The low energy dissociation technique electron capture dissociation has been applied to a series of thrombospondin and properdin derived O-fucosylated glycopeptides. Followed by capture of an electron by multiply protonated precursor ions $[M + nH]^{n+}$ reduced odd electron radical cations $[M + nH]^{(n-1)\bullet+}$ were generated. The latter mainly fragment by cleavage of the N–Cα bonds of the peptide chain without loss of the labile sugar moiety allowing an unambiguous assignment of the glycosylation site. Apart from peptide backbone cleavages, side chain losses of aminocarbonylmethyl and aminocarbonylmethylthiyl radicals from carboxyamidomethylated cysteins are observed. The N–Cα bond cleavage is greatly reduced on both sides of alkylated Cys. However, fragment ions that are formed by secondary fragmentations of z-type radical cations containing N-terminal cystein give rise to even electron z–$^{\bullet}SCH_2CONH_2$ ions. The potential of the high mass accuracy for the identification of the protein modification topology has been fully explored.

Keywords: ECD; Glycopeptide; Fucosylation; Mannosylation

1. Introduction

Fucose is commonly present as a terminal sugar residue at the non-reducing end of N- and O-glycans in glycoproteins and glycosphingolipids of eukaryotic cells [1,2]. In contrast, O-fucosylation is an unusual type of protein glycosylation, where fucose is attached directly through O-glycosidic bond to either serine or threonine in the polypeptide chain. This modification was previously thought to occur exclusively within epidermal growth factor (EGF) domains of a few proteins, in form of a monosaccharide, or elongated by *N*-acetylglucosamine, galactose and sialic acid to a tetrasaccharide structure [3]. We have recently identified and structurally characterized a new type of O-fucosylation within thrombospondin type-1 repeats (TSR), where the O-fucose residue is elongated by a glucose to a disaccharide structure O-Fuc-Glc [4]. This modification appears to be common to TSR, a structural motif present in many protein families, which points to the fact that much larger number of proteins may be O-fucosylated than previously thought [5].

There is growing number of reports indicating that O-fucosylation plays a significant role in function of modified proteins, especially in signal transduction processes. Effects of O-fucosylation were extensively studied in the Notch signalling pathway [6,7]. Silencing of O-fucosyltransferase I (O-FucT-1) gene in *Drosophila* was shown to abolish almost all aspects of Notch signalling [7], whereas O-FucT-1 knock-out mice were shown to die at mid-gestation [8]. Therefore, O-FucT-1 was proposed to be an essential member in the Notch signalling pathway. In addition, O-fucosylation has been reported to play a role in Nodal [9] and urokinase-plasminogen activator receptor (u-PAr) signalling pathways [10]. The exact structural role of O-fucosylation in signal transduction has not yet been elucidated.

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The structural analysis of glycopeptides and glycoproteins by mass spectrometric methods still remains a somewhat elusive goal. Several approaches have been introduced to study both the glycan and the protein backbone [11,12]. The direct determination of glycosylation sites in O-glycopetides is hampered by the lability of the glycosidic bonds, i.e. loss of the glycan usually occurs faster than the cleavage of the amide bonds when ergodic fragmentation processes are considered. However, it has been shown that under special conditions diagnostic low abundant glycosylated b- and y-type ions can be obtained by use of post-source decay (PSD) and collision-induced dissociation (CID) [13–15].

In studies of posttranslation modifications of TSP-1, mass spectrometry was for the first time used for complete characterization of protein O-fucosylation. TSP-1-derived glycopeptides were submitted to CID in a nano-ESI Q-TOF instrument under conditions that enabled direct determination of O-fucosylation status, glycan structure and O-fucosylation sites in a single spectrum [16].

During the last years electron capture dissociation (ECD) rapidly evolved as an alternative activation method especially in the analysis of peptides and proteins [17–41]. By irradiation of multiply charged analyte ions $[M+nH]^{n+}$ produced by electrospray ionization [42] with low energy electrons ($T \leq 0.2$ eV), reduced radical cations $[M+nH]^{(n-1)\bullet+}$ are generated. These odd electron species dissociate mainly by fast and facile fragmentation of the N–Cα bonds of the peptide chain giving rise to mainly enolimine (c-type) and aminoketyl radical ($z^{\bullet}$-type) ions. This process has been suggested to be "non ergodic", i.e. bond cleavage prior to distribution of the excess energy gained from the charge recombination event into all vibrational modes of the reduced species. The non-ergodic nature and the mechanism of ECD is still a matter of debate in the current literature and evidence has been reported supporting this concept [18,21,39,43–46].

Hence, ECD has developed into a powerful low energy activation method giving rise to sequence information of peptides and intact proteins. Also, application of ECD to the analysis of other species such as peptide nucleic acids [47], polyglycols [48–50], polyesteramides [44], aminosugars [51,52] or nucleotides [53] has been reported.

As a consequence of the fast and facile fragmentation of the N–Cα bonds under ECD conditions the elimination of labile substituents such as glycans or phosphoryl groups from the peptide backbone obviously cannot compete with the backbone cleavage within the radical cations. This approach can therefore be used for an unambiguous assignment of the attachment sites of these modifications within the peptide chain. Up to now the enormous potential of this method has been demonstrated for the analysis and characterization of modified peptides [54]. These include peptide O- and N-glycosylation [55–62], phosphorylation [61,63], sulphation [64], γ-carboxylation [64,65], fatty acid modifications [66] and methionine oxidations [67]. Furthermore, ECD has been applied to the examination of non-covalent interactions and weakly bound complexes [56,68–70]. Recently, also ECD-type fragmentations in noncovalent protein complexes have been reported that are induced by electron transfer initiated by charge partitioning [71].

In the present contribution, we demonstrate how information obtained from ECD spectra can be used to characterize O-fucosylated peptides derived from thrombospondin-1 and properdin.

2. Experimental methods

2.1. Samples

Four O-fucosylated glycopeptides were used in nano-ESI ECD FT-ICR MS experiments. Peptides SSCSV$\underline{\underline{T}}$CGDGVI TR, **T^{1}**, with O-Fuc-Glc at Thr 6 and TSCST$\underline{\underline{S}}$CGNGIQQR, **T^{2}**, with O-Fuc-Glc at Ser 6 were obtained by enzymatic digestion of thrombospondin-1 and purified as described previously [4]. Glycopeptides TPCSASCHGGPHEPK, **P^{1}**, with O-Fuc-Glc at Ser 6 and WSLWSTWAPCSVTCSE, **P^{2}**, with O-Fuc-Glc at Thr 13, as well as with a single mannose residues on Trp4 and Trp7, were purified from enzymatic digests of human properdin. Preparation of these peptides will be described elsewhere (Gonzalez de Peredo et al., unpublished results).

2.2. Electron capture dissociation FT-ICR mass spectrometry

ECD experiments were performed by use of an APEX II FT-ICR mass spectrometer (Bruker Daltonics, Billerica, MA) equipped with a 9.4 T superconducting magnet (Magnex Scientific Ltd., Oxford, UK) and an Infinity^{TM} cell [72]. Gas-phase ions were generated by nano-electrospray ionization by use of an Apollo ESI source. The O-glycopeptide stock solutions were diluted to final concentrations of approx. two to five pmol μl^{-1} into electrospray solutions consisting of methanol/water/acetic acid (49.5/49.5/1, v/v/v). Typical source parameters were capillary voltages of 800 V and cone voltages of 65 V. Ions were accumulated in the hexapole located behind the second skimmer of the ion source for 0.5–3 s and then transferred into the cell of the ICR instrument. In experiments where ECD was applied to the glycosylated peptide ions derived from **P^{1}** and **P^{2}** a conventional Rhenium filament was used. Trapping was achieved without "sidekick" by gas-assisted dynamic trapping at potentials of 4 V followed by a pumping delay of 0.5 s. Argon was used as a collision gas. The trapped multiply charged precursor ions were isolated by standard ejection procedures to eliminate ions except those of interest by a broad band r.f. pulse and a series of r.f. pulses with the cyclotron frequencies close to that of the selected ion ("single shots"), in order to prevent unintended excitation. Then the trapping voltages were lowered to 1 V by use of a pre-programmed voltage ramp. Subsequently, the peptide ions were irradiated with low energy electrons, emitted by

an unmodified Rhenium filament located 10 cm behind the rear trapping plate of the ICR cell for 10 s. Typically, filament currents of 3.0 A and acceleration voltages of 1.7 V were used. In three experiments, namely ECD of the multiply protonated precursor ions derived from **T¹**, **T²** and **P²**, an indirectly heated dispenser cathode (6 mm diameter, tungsten doped with barium oxide, HeatWave, Watsonville, CA, USA) was used as an electron source. Trapping was achieved without application of gas-assisted dynamic trapping but by use of a "sidekick", which was shown to be appropriate for this experimental setup for both ECD and IRMPD as shown previously [29]. Since the pumping delay for the removal of the gas is no longer required, the time for a single scan is significantly reduced. After isolation of the desired precursor ions (vide supra) low energy electrons (∼1 eV) were allowed to enter the ICR cell for 250 ms. The heater current of the indirectly heated dispenser cathode was set to 1.8 A (7.8 V) by use of an external power supply. All mass spectra were acquired in the broadband mode in the mass range from *m/z* 200 to 2600 with 512 k data points. The time-domain signals were zero filled quadruply to enhance the digitization of the spectrum and to improve its visualization [73], followed by apodization with a quadratic sine bell function prior to Fourier transformation. For all spectra shown 32 scans were accumulated. The ECD spectra were internally calibrated for one species under inspection on the $[M+nH]^{n+}$ and $[M+(n-1)H]^{(n-1)+}$ ions and their respective isotopomers. This calibration was used as an external calibration for each respective set of experiments. By use of this external calibration most spectra provided a mass accuracy better than 7 ppm. The majority of unassigned peaks correspond to noise spikes, foldback peaks, or bleedthrough from the isolation waveform. Only peaks that exhibited a sufficient signal to noise ratio to observe the corresponding isotopomeric peaks were included in the assignments. Acquisition and data processing was performed with the Bruker Daltonics software XMass (version 6.0.0). The experiments were controlled by pulse programs written in-house.

3. Results and discussion

3.1. Electron capture dissociation of O-fucosylated peptide ions derived from thrombospondin

The ECD spectrum of the doubly protonated thrombospondin derived glycopeptide ions $[\mathbf{T^1}+2H]^{2+}$ is shown in Fig. 1 and the corresponding mass assignments are given in Table 1.

C-terminal z-type ions are observed exclusively with a mass accuracy of 9.9 ppm or higher. The observed fragmentation pattern can be explained by the fact that the C-terminal residue is an arginine which is by far the most basic site within the peptide chain and therefore will be one of the most favourable protonation sites [74,75]. Earlier observations by Håkansson et al. suggest the preferred incorporation of such

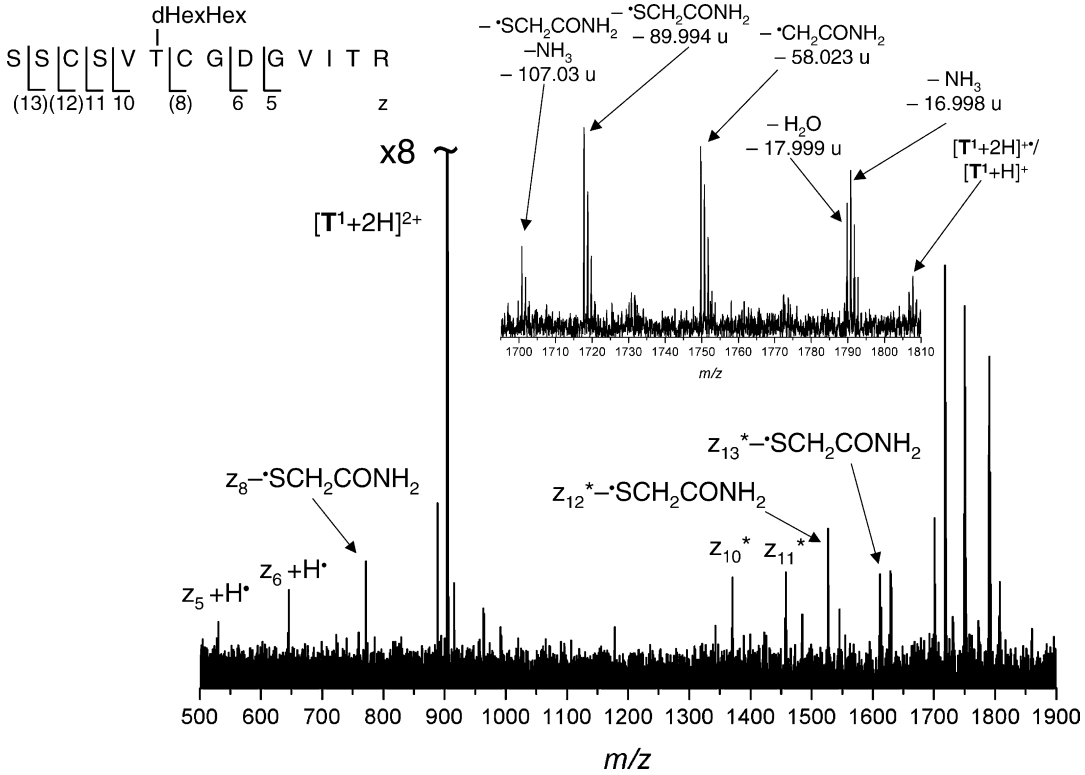


Fig. 1. Electron capture dissociation FT-ICR mass spectrum obtained from the doubly protonated thrombospondin-1 derived O-fucosylated glycopeptide ions $[\mathbf{T^1}+2H]^{2+}$.

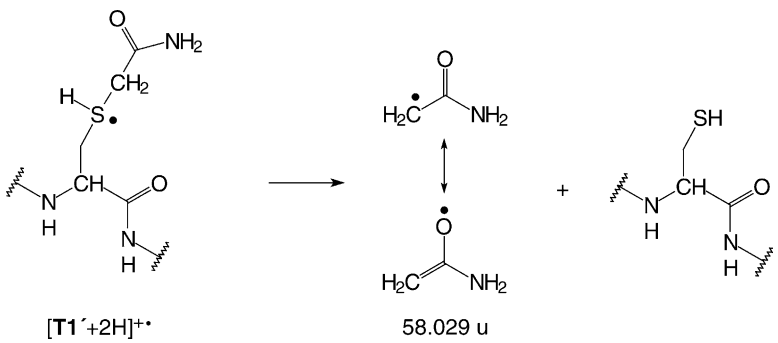
Table 1
Ions observed after ECD of the doubly protonated O-fucosylated thrombospondin-1 derived peptide ions $[\mathbf{T^1} + 2H]^{2+}$

Assignment	Observed *m/z*	Theoretical *m/z*	Error (ppm)
$z_5 + 1H^\bullet$	530.3247	530.3295	−9.1
$z_6 + 1H^\bullet$	645.3511	645.3566	−8.5
z_8–$^\bullet SCH_2CONH_2$	771.3922	771.3995	−9.3
$[\mathbf{T^1} + 2H]^{2+}$	903.8862	903.8902	−4.4
$z_{10}^*/z_{10}^* + 1H^\bullet$	1369.629/1370.636	1369.628/1370.635	+0.7/+0.7
$z_{11}^*/z_{11}^* + 1H^\bullet$	1456.660/1457.674	1456.659/1457.667	+0.7/−2.0
z_{12}^*–$^\bullet SCH_2CONH_2$	1526.692	1526.689	+2.0
z_{13}^*–$^\bullet SCH_2CONH_2$	1613.729	1613.721	+4.9
$[\mathbf{T^1} + 2H$–$^\bullet SCH_2CONH_2$–$NH_3]^+$	1700.757	1700.756	+0.6
$[\mathbf{T^1} + 2H$–$^\bullet SCH_2CONH_2]^+$	1717.796	1717.779	+9.9
$[\mathbf{T^1} + 2H$–$^\bullet CH_2CONH_2]^+$	1749.763	1749.753	+5.7
$[\mathbf{T^1} + 2H$–$H_2O]^{\bullet+}$	1789.796	1789.770	+14.0
$[\mathbf{T^1} + 2H$–$NH_3]^{\bullet+}$	1790.770	1790.757	+7.2
$[\mathbf{T^1} + 1H]^+$	1806.769	1806.773	−2.2
$[\mathbf{T^1} + 2H]^{\bullet+}$	1807.787	1807.781	+3.3

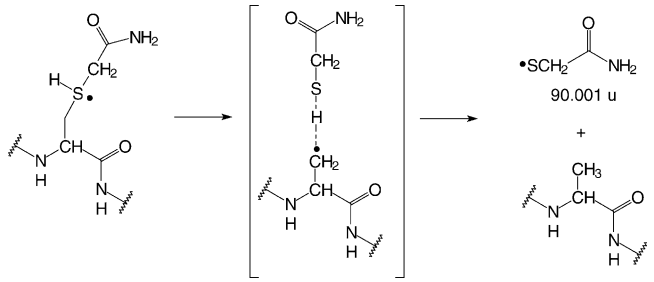
* Denotes fully glycosylated fragment ions.

protonated basic residues in the charged fragment ions [25]. Therefore, all c-type fragments will be neutral if the precursor ions are doubly protonated. The ECD spectrum of the $[\mathbf{T^1} + 2H]^{2+}$ ions exhibits four glycosylated z*-type ions (the asterisk denotes glycosylated fragment ions) or z*-type derived ions (vide infra) and three z-type ions without glycosylation, some of them shifted by ∼1 u as a consequence of $H^\bullet$-transfer processes [18,31]. Even if the complete amino acid sequence cannot be derived from the ECD spectrum these information are sufficient to unambiguously assign the glycosylation site of the species. According to the fragmentation observed the only real O-glycosylation site can be the threonine residue at position 6 counted from the N terminus. Apart from major backbone fragment ions also ionic species arising from the loss of smaller neutral fragments give rise to intense signals in the *m/z* region below the reduced singly charged molecular ions $[\mathbf{T^1} + 2H]^{\bullet+}$ (cf. insert Fig. 1). In several studies on ECD of both linear and cyclic peptide ions such small neutral losses from amino acid side chains have been reported [17,39–41]. As expected, a single hydrogen atom is expelled from the reduced species commonly found in the ECD spectra of small size peptide ions. Also the elimination of ammonia (loss of 16.998 u) and water (loss of 17.999 u) is observed. The former fragmentation appears most likely upon neutralisation by electron capture from either the protonated N terminus or from the arginine present in the peptide. The H_2O loss is most probably due to partial proton solvation of the hydroxyl group of a threonine side chain and subsequent radical site-induced elimination as outlined recently by Haselmann et al. [41]. Less likely is the loss of water from serine residues, which would generate unstable primary radicals. Furthermore, fragment ions at *m/z* 1749.663, 1717.796 and 1700.757 corresponding to losses of 58.023 u (C_2H_4ON), 89.994 u (C_2H_4ONS) and 107.03 u ($C_2H_7ON_2S$) are observed (for theoretical masses cf. Table 1). These can be attributed to the elimination of odd electron species from the carboxyamidomethylated side chains of the cystein residues. It has been shown that mono- and disulphide bonds are preferentially cleaved under ECD conditions [18,19,76]. This observation has been explained by the hot hydrogen-atom mechanism suggesting a $H^\bullet$ transfer to a site with high hydrogen affinity, i.e. a disulphide bridge or sulphide sulphur. The subsequent fast rupture of these bonds is due to the radical site formed by irreversible hydrogen attachment. It is likely to assume a similar mechanism in this particular case (cf. Schemes 1 and 2).

The radical cations $[\mathbf{T^{1'}} + 2H]^{\bullet+}$ fragment by cleavage of either one of the S–C bonds adjacent to the hypervalent sul-



Scheme 1.

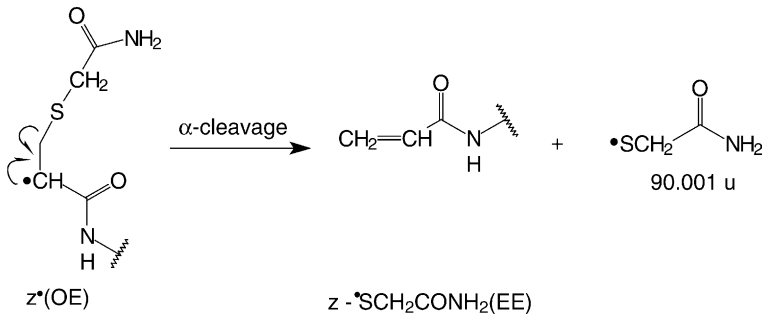


Scheme 2.

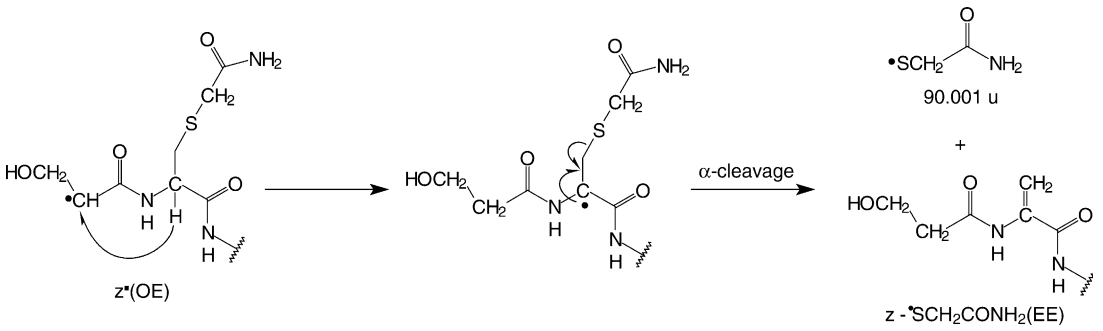
phur. The S–C 0 K bond dissociation energies in hypervalent sulphur centred radicals formed by attachment of a H• atom to dimethyl sulphide are remarkably low (52 kJ mol^{-1}) as revealed by ab initio calculations reported by Tureček et al. [77]. Radical site-induced fragmentation gives rise to resonance stabilized aminocarbonylmethyl radicals and even electron singly charged fragment ions at *m*/*z* 1749.763. On the other hand, cleavage of the S–C bond attached to the peptide leads to the formation of aminocarbonylmethylthiyl radicals (cf. Scheme 2). Since this fragmentation requires a hydrogen shift from the sulphur to the adjacent methylene group we assume an intermolecular H•-abstraction from the departing thiol to the initially formed radical within an ion/molecule complex rather than a concerted S–C bond cleavage accompanied by an unfavourable 1,2-H•-shift [78]. Furthermore, loss of aminocarbonylmethylthiyl radicals accompanied by ammonia loss is observed (*m*/*z* 1700.757). The fragmentation processes outlined above are obviously specific for carboxyamidomethylated cystein residues which are routinely formed when standard protocols for reductive S–S bond cleavages preceding peptide digests are applied [79–82]. Another remarkable observation with respect to cystein alkylation is the low abundance or non-occurrence of fragmentations at Cys especially towards the N terminus. This reduced cleavage has already been reported for unprotected cystein residues which were found to block the formation of both c- and z-type fragments [33]. In this case, the loss of C_2H_4ONS from the C-terminal z•-type ions resulting into even electron z-•SCH_2CONH_2 fragment ions as secondary fragments is observed. The most likely mechanism to explain this finding is shown in Scheme 3.

The initially formed z•-type radical cations fragment by α-cleavage and formation of an α,β-unsaturated carbonyl species and the relatively stable thiyl radicals. This mechanism is corroborated by the observation that no loss of aminocarbonylmethyl radicals from the z•-type radical cations is observed. Therefore, an initial H• transfer to the sulphide bond and fragmentation of the corresponding hypervalent sulphonium radicals can be excluded. The occurrence of z_{13}–•SCH_2CONH_2 fragment ions, i.e. fragmentation towards the N terminus one residue away from the Cys and subsequent loss of the thiyl radical, might be explained by a—formally allowed—1,4-H•-shift from the α-CH-group of the cystein residue to the initially formed terminal secondary z_{13} radical fragment again followed by an α-cleavage giving rise to a dehydroalanine residue (cf. Scheme 4). Again, no loss of aminocarbonylmethyl radicals from the z•-type radical cations is observed, therefore, the formation of a sulphur centred radical species is unlikely. This mechanistic hypothesis is corroborated by the observation that the αC–H bond dissociation energies in peptides were found to be relatively low (<370 kJ mol^{-1} [83,84]).

The second thrombospondin derived O-glycopeptide (**T^2**) examined should contain an Asn residue at position 9. In contrast to this expectation MS^1 as well as the ECD spectra of this sample reveal the presence of a mixture of two distinct species. Obviously, the amide group of Asn has been hydrolysed to aspartic acid by non enzymatic degradation as reported earlier for the Asn–Gly motif and therefore a mass shift of ∼1 u is observed [85–87]. Examination of the broadband MS^1 spectrum exhibits this modification unambiguously. In Fig. 2 enlarged views of the *m*/*z* regions of



Scheme 3.



Scheme 4.

the doubly and triply protonated pseudo molecular ions are shown.

The $[\mathbf{T^2} + 2H]^{2+}$ ions containing the N residue give rise to a signal of a minor relative intensity at *m/z* 932.3895 as predicted from the aa sequence (Fig. 2A). The base peak of the spectrum is produced by $[\mathbf{T^{2\#}} + 2H]^{2+}$ ions containing the D residue at *m/z* 932.8790. The observed pattern already indicates the presence of two species differing in mass by ∼1 u.

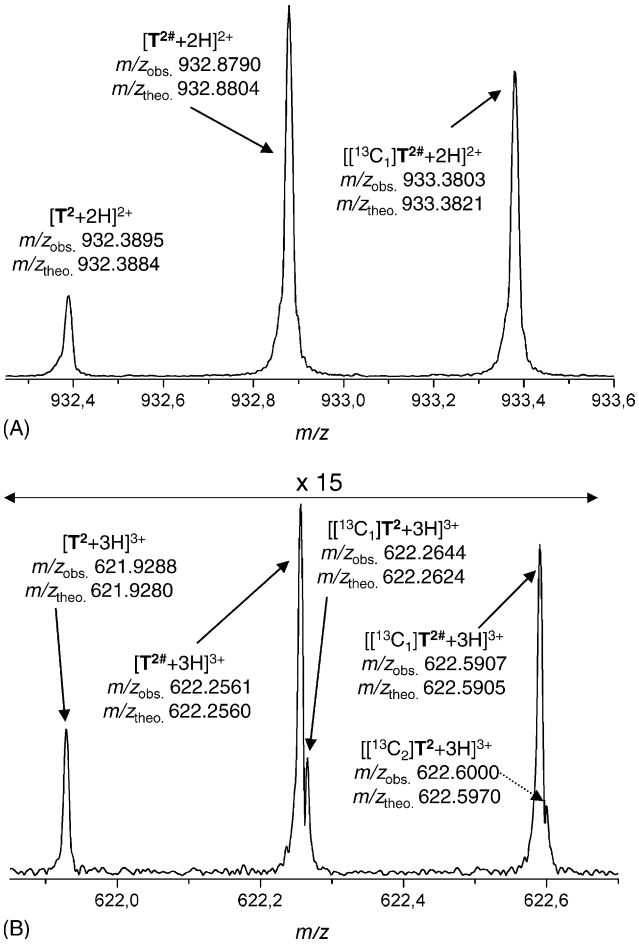


Fig. 2. Mass scale expansions near *m/z* 932 (A) and *m/z* 622 (B) of the broadband positive nano-ESI FT-ICR spectrum of the thrombospondin-1 derived O-fucosylated glycopeptide mixture $\mathbf{T^2}$ and $\mathbf{T^{2\#}}$.

Under the present conditions the resolution at *m/z* 932 is not sufficient to separate the $[\mathbf{T^{2\#}} + 2H]^{2+}$ ions from the peak corresponding to the isobaric $[^{13}C_1]$ $[\mathbf{T^2} + 2H]^{2+}$ ions [88]. A sufficient resolution can be achieved in the *m/z* region of the triply protonated peptide ions giving rise to far less intense signals. As shown in Fig. 2B the isobaric $[\mathbf{T^{2\#}} + 2H]^{3+}/[^{13}C_1][\mathbf{T^2} + 2H]^{3+}$ ions can be distinguished. Therefore, all ECD fragments containing amino acid residue 9 (counted from the N terminus) exhibit a doublet in the spectrum.

The ECD spectrum of the doubly protonated thrombospondin derived glycopeptide ions $[\mathbf{T^2}/\mathbf{T^{2\#}} + 2H]^{2+}$ is shown in Fig. 3 and the corresponding mass assignments are given in Table 2.

Again, C-terminal $z^\bullet$-type ions are observed exclusively with a mass accuracy of 6.2 ppm or higher. In contrast to the ECD spectrum of the $[\mathbf{T^1} + 2H]^{2+}$ precursor ions an almost complete set of z-type ions are detected, i.e. 11 out of 13 possible cleavages. Also the glycosylation site can be assigned unambiguously. As expected all fragment ions containing aa-residue 9 (N/D) give rise to two signals separated by 0.983 u. Also the fragmentation pathways associated with the carboxyamidomethylated cystein residue discussed above are observed. Loss of aminocarbonylmethyl and aminocarbonylmethylthiyl radicals gives rise to fragment ion signals. Also the $z^\bullet$-type radical cations eliminate $^\bullet SCH_2CONH_2$ radicals if the N-terminal amino acid is Cys.

Additionally, the unintended loss of the sugar moiety from the doubly charged precursor ions is observed. This elimination is most probably due to either collisional-induced dissociation of excited ions formed by the isolation procedure colliding with the background gas [31] or by fragmentation of ions within the time scale of the experiment still containing vibrational excess energy from the ionization process [59].

3.2. Electron capture dissociation of O-fucosylated peptide ions derived from properdin

In a second set of experiments, the properdin derived peptides $\mathbf{P^1}$ and $\mathbf{P^2}$ were submitted to ECD. The ECD spectrum obtained from triply protonated precursor ions $[\mathbf{P^1} + 3H]^{3+}$ is shown in Fig. 4, the corresponding assignments of the de-

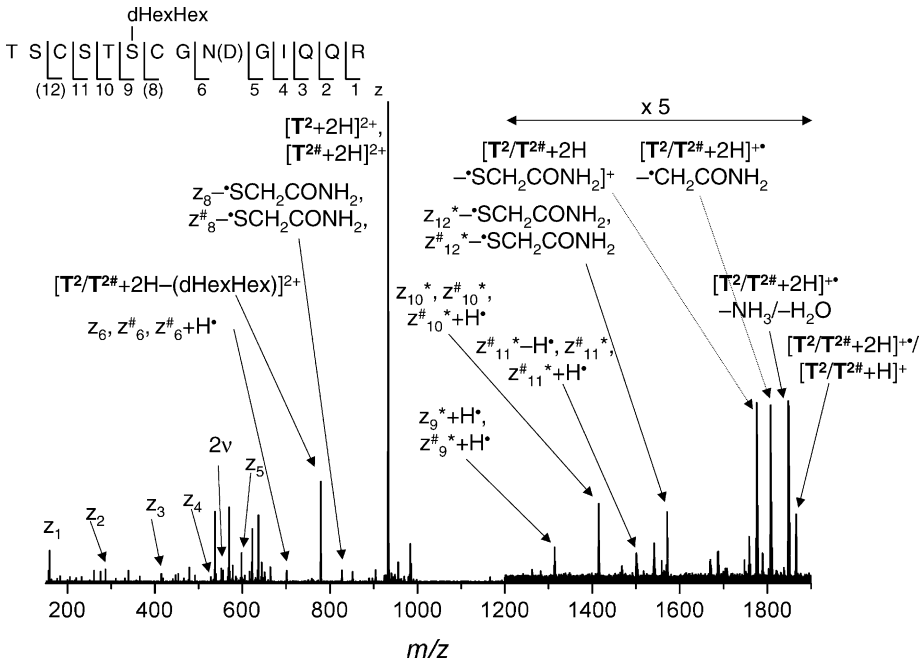


Fig. 3. Electron capture dissociation FT-ICR mass spectrum obtained from the doubly protonated thrombospondin-1 derived O-fucosylated glycopeptide ion mixture $[\mathbf{T^2} + 2H]^{2+}$ and $[\mathbf{T^{2\#}} + 2H]^{2+}$.

tected ions together with their measured and calculated *m/z* values are given in Table 3.

Apart from capture of one electron to yield $[\mathbf{P^1} + 3H]^{\bullet 2+}$ ions also the formation of the even electron $[\mathbf{P^1} + 3H]^{+}$ ions arising from reduction by two electrons is observed.

Eight even electron c-type fragment ions and one $z^{\bullet}$-type radical cation species give rise to peaks in the spectrum. The observed fragment ions correspond to fragmentation of eight out of fourteen amide bonds. Since the sequence of $\mathbf{P^1}$ contains two prolines, cleavages on the N-terminal side of

Table 2
Ions observed after ECD of the doubly protonated O-fucosylated thrombospondin-1 derived O-fucosylated glycopeptide ion mixture $[\mathbf{T^2} + 2H]^{2+}$ and $[\mathbf{T^{2\#}} + 2H]^{2+}$

Assignment	Observed *m/z*	Theoretical *m/z*	Error (ppm)
z_1	159.0997	159.1001	−2.5
z_2	287.1579	287.1587	−2.8
z_3	415.2164	415.2172	−1.9
z_4	528.3006	528.3013	−1.3
z_5	585.3217	585.3228	−1.9
$z_6/z_6^{\#}/z_6^{\#} + 1H^{\bullet}$	699.3656/700.3488/701.3567	699.3657/700.3497/701.3575	−0.1/−1.3/−1.2
$[\mathbf{T^2} + 2H–(dHexHex)]^{2+}/[\mathbf{T^{2\#}} + 2H–(dHexHex)]^{2+}$ [a]	778.3362/778.8237	778.3330/778.8250	+3.9/−1.6
$z_8–^{\bullet}SCH_2CONH_2/z_8^{\#}–^{\bullet}SCH_2CONH_2$	826.4157/827.3997	826.4165/827.4005	−1.0/−1.0
$[\mathbf{T^2} + 2H]^{2+}/[\mathbf{T^{2\#}} + 2H]^{2+}$	932.3883/932.8778	932.3884/932.8804	−0.1/+2.8
$z_9^* + 1H^{\bullet}/z_9^{*\#} + 1H^{\bullet}$	1312.576/1313.551	1312.568/1313.552	+6.1/−0.9
$z_{10}^*/z_{10}^{*\#}/z_{10}^{*\#} + 1H^{\bullet}$	1412.609/1413.589/1414.602	1412.608/1413.592/1414.600	+0.7/−1.7/+1.2
$z_{11}^{*\#}–1H^{\bullet}/z_{11}^{*\#}/z_{11}^{*\#} + 1H^{\bullet}$	1449.622/1500.625/1501.634	1499.616/1500.624/1501.632	+3.4/+0.7/+1.3
$z_{12}^*–^{\bullet}SCH_2CONH_2/z_{12}^{*,\#}–^{\bullet}SCH_2CONH_2$	1569.676/1570.659	1569.669/1570.654	+4.5/+3.2
$[\mathbf{T^2} + 2H–^{\bullet}SCH_2CONH_2]^{+}/[\mathbf{T^{2\#}} + 2H–^{\bullet}SCH_2CONH_2]^{2+}$	1774.787/1775.773	1774.776/1775.760	+6.2/+7.3
$[\mathbf{T^2} + 2H–^{\bullet}CH_2CONH_2]^{+}$	1806.755	1806.748	+3.8
$[\mathbf{T^{2\#}} + 2H–^{\bullet}CH_2CONH_2]^{2+}$	1807.738	1807.732	+3.3
$[\mathbf{T^{2\#}} + 2H–H_2O]^{\bullet +}$	1846.775	1846.767	+4.3
$[\mathbf{T^2} + 2H–NH_3]^{\bullet +}/[\mathbf{T^{2\#}} + 2H–H_2O]^{\bullet +}$	1847.761	1847.754/1847.751	+3.9/+5.4
$[\mathbf{T^{2\#}} + 2H–NH_3]^{\bullet +}$	1848.748	1848.738	+5.4
$[\mathbf{T^2} + 1H]^{+}$	1863.762	1863.770	−4.3
$[\mathbf{T^2} + 2H]^{\bullet +}$	1864.775	1864.777	−1.1
$[\mathbf{T^{2\#}} + 2H]^{\bullet +}$	1865.774	1865.761	+7.0

[a] dHexHex = FucGlc.

* Denotes fully glycosylated fragment ions.

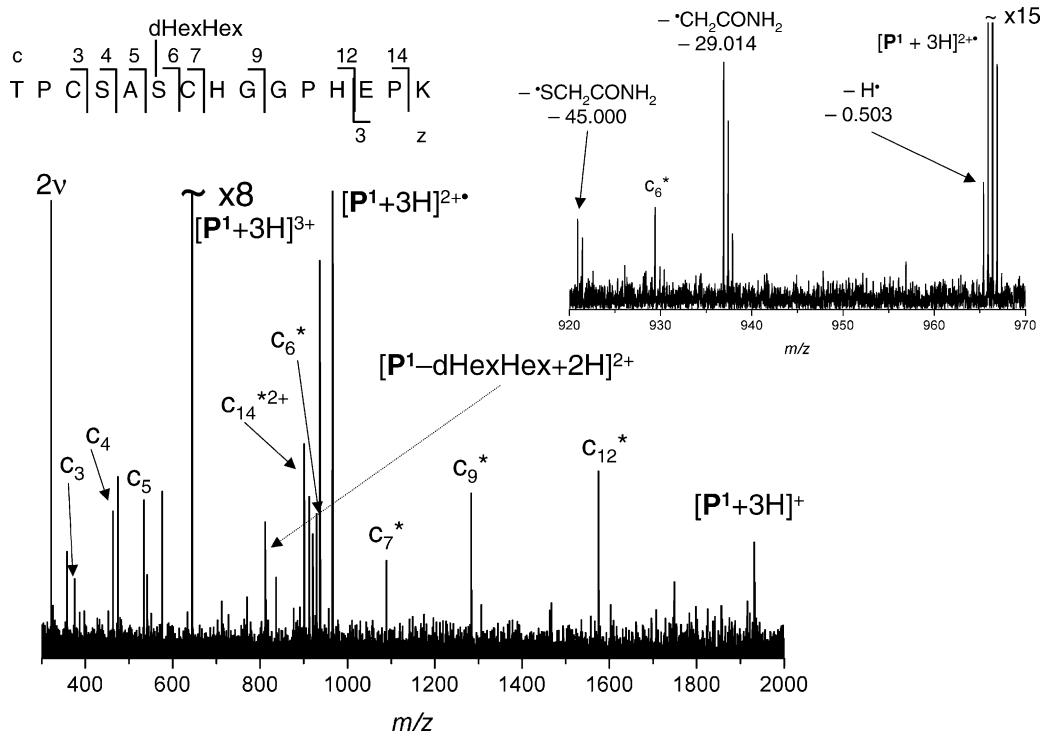


Fig. 4. Electron capture dissociation FT-ICR mass spectrum obtained from the triply protonated properdin derived O-fucosylated glycopeptide ions $[\mathbf{P^1} + 3H]^{3+}$.

these residues cannot be expected due to the cyclic structure of proline requiring rupture of two bonds [17]. No loss of the glycan moiety from the fragment ions is observed whereas a low level of deglycosylation occurs from the $[\mathbf{P^1} + 3H]^{3+}$ precursor ions. These sequence information are sufficient to assign the glycosylation site. Also the radical site-induced elimination of aminocarbonylmethyl and aminocarbonylmethylthiyl radicals from the doubly charged $[\mathbf{P^1} + 3H]^{\bullet 2+}$ ions are observed (cf. insert Fig. 4). In contrast to the thrombospondin derived O-glycopeptides discussed before, $\mathbf{P^1}$ contains a C-terminal lysine residue instead of arginine and two histidine residues within the sequence. This structural difference might be an explanation for the preferred formation of N-terminal c-type fragment ions. The gas-phase basicities of lysine (GB = 929 kJ mol^{-1}) and histidine (GB = 936 kJ mol^{-1}), have been determined to be significantly lower than the GB of arginine (GB = 992 kJ mol^{-1}) [74,75]. The intrinsic basicity of the C-terminal residue is obviously not high enough to retain the charge such as does arginine. Therefore, especially if stabilization by charge solvation is taken into account [41], there is a higher probability of a somewhat more statistical distribution of the initial protonation sites. In contrast to the thrombospondin derived species no loss of aminocarbonylmethylthiyl radicals from c-type fragment ions is observed corroborating the radical site-induced α-cleavage from the odd electron z$^\bullet$-type radical cations.

Table 3
Ions observed after ECD of the triply protonated properdin derived O-fucosylated glycopeptide ions $[\mathbf{T^1} + 3H]^{3+}$

Assignment	Observed *m/z*	Theoretical *m/z*	Error (ppm)
z_3	357.191	357.189	+6.5
c_3	376.167	376.165	+5.3
c_4	463.200	463.197	+6.5
c_5	534.237	534.234	+5.6
$[\mathbf{P^1} + 3H]^{3+}$	643.936	643.937	−1.6
$[\mathbf{P^1} + 2H–(dHexHex)]^{2+}$ [a]	811.852	811.851	+1.2
$c_{14}{}^{*2+}$	900.861	900.862	−1.1
$[\mathbf{P^1} + 3H–{}^\bullet SCH_2CONH_2]^{2+}$	920.906	920.905	+1.1
$c_6{}^*$	929.383	929.377	+6.5
$[\mathbf{P^1} + 3H–{}^\bullet CH_2CONH_2]^{2+}$	936.892	936.891	+1.1
$[\mathbf{P^1} + 2H]^{2+}$	965.403	965.401	+2.1
$[\mathbf{P^1} + 3H]^{\bullet 2+}$	965.906	965.905	+1
$c_7{}^*$	1089.413	1089.408	+4.6
$c_9{}^*$	1283.491	1283.488	+2.3
$c_{12}{}^*$	1574.624	1574.621	+1.9
$[\mathbf{P^1} + 3H]^{+}$	1931.807	1931.810	+1.5

[a] dHexHex = FucGlc.
* Denotes fully glycosylated fragment ions.

We have also examined the fragmentation of a C-glycosylated peptide, i.e. mannosylation at the C-2 position of the indol side chain of tryptophan [4]. The species under inspection, $\mathbf{P^2}$, contains two mannosyltryptophanes in positions 4 and 7 and a fucosylation at the threonine in position 13. The ECD spectrum of the doubly protonated properdin derived glycopeptide ions $[\mathbf{P^2} + 2H]^{2+}$ is shown in Fig. 5 and the corresponding mass assignments are given in Table 4.

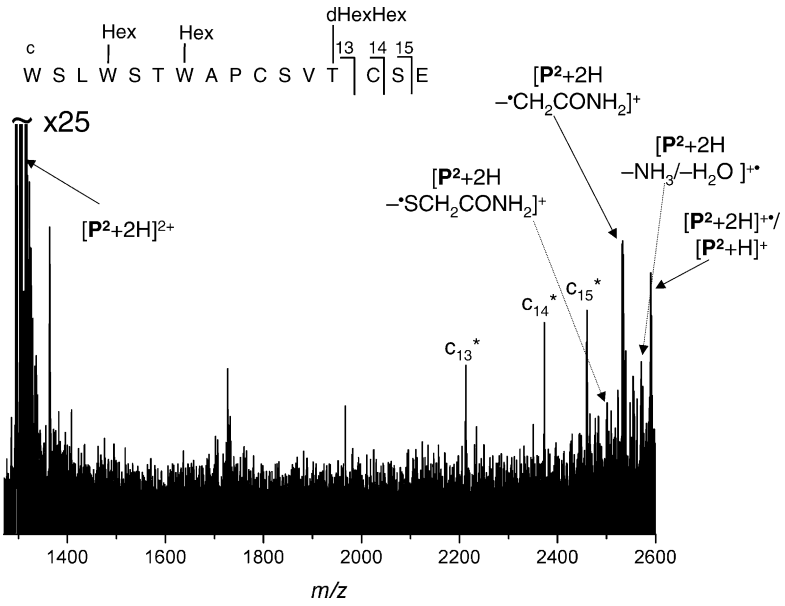


Fig. 5. Electron capture dissociation FT-ICR mass spectrum obtained from the doubly protonated properdin derived O-fucosylated and C-mannosylated glycopeptide ions $[\mathbf{P^1} + 3H]^{3+}$.

Apart from the loss of some small neutral species only some N-terminal c-type fragment ions of minor abundance were detected. Interestingly, exclusive cleavage of the last three C-terminal amide bonds is induced upon EC, while other amide bonds towards the N terminus following the O-fucosylation site remain intact. As expected, neither the elimination of the dHexHex glycan nor the $^{0,2}X_0$ ring cleavage—found typically upon collisional excitation of mannosylated even electron precursor ions [4,89]—were observed under ECD conditions. The spectrum depicted in Fig. 5 exhibits a rather poor signal to noise ratio. However, several attempts to improve the EC efficiency, such as by resonant excitation of the precursor ions [62], by use of an indirectly heated dispenser cathode [32], by variation of the electron irradiation time, by increasing the amount of $[\mathbf{P^2} + 2H]^{2+}$ ions by an increased external accumulation time [56] and also by vibrational excitation of the precursor ions using different source conditions did not deliver spectra containing more structural information. A priori an enhanced fragmentation on the C-terminal side of the three Trp residues as a result of the high $H^\bullet$ affinity reported earlier was expected [19,33]. Neither one of these bonds were cleaved, which is most likely due to the fact that the C-2 position is no longer accessible for a hydrogen attack due to steric hindrance by the glycan substituent. Also side chain losses that have been observed for Trp [41] were here not detected most probably for the same argument. Similar reduced fragmentation frequencies were observed when other C-mannosylated species were submitted to ECD (data not shown).

Table 4
Ions observed after ECD of the doubly protonated properdin derived O-fucosylated and C-mannosylated glycopeptide ions $[\mathbf{P^1} + 3H]^{3+}$

Assignment	Observed *m/z*	Theoretical *m/z*	Error (ppm)
$[\mathbf{P^2} + 2H]^{2+}$	1295.024	1295.025	−0.9
$c_{13}{}^*$	2211.948	2211.953	−3.0
$c_{14}{}^*$	2372.001	2371.983	+7.6
$c_{15}{}^*$	2459.019	2459.015	+1.6
$[\mathbf{P^2} + 2H–{}^\bullet SCH_2CONH_2]^+$	2500.057	2500.048	+3.6
$[\mathbf{P^2} + 2H–{}^\bullet CH_2CONH_2]^+$	2532.029	2532.022	+2.8
$[\mathbf{P^2} + 2H–H_2O]^{\bullet+}$	2572.051	2572.039	+4.7
$[\mathbf{P^2} + 2H–NH_3]^{\bullet+}$	2573.043	2573.026	−6.6
$[\mathbf{P^2} + 2H]^{\bullet+}$	2590.070	2590.049	+8.1

* Denotes fully glycosylated fragment ions.

4. Conclusion

In summary, the results outlined in this study show that ECD represents a valuable approach to the structural elucidation of O-fucosylated peptide ions derived from thrombospondin and properdin. Apart from amino acid sequence information the glycosylation site can be assigned unambiguously. In contrast to O-fucosylated peptide ions C-mannosylations within the peptide chain obviously hamper backbone cleavages induced by electron capture.

Additionally, radical site-induced loss of aminocarbonylmethyl and aminocarbonylmethylthiyl radicals from carboxyamidomethylated cystein side chains were observed. These are most likely induced by hydrogen attachment to the sulphide bridge to form hypervalent sulphur centred radicals. Furthermore, secondary fragmentations of $z^\bullet$-type radical cations with an N-terminal alkylated cystein residue eliminating aminocarbonylmethylthiyl radicals by α-cleavage are observed. These fragmentation processes

appear to be specific for carboxyamidomethylated cystein residues and therefore they might have a somewhat diagnostic use. Since carboxymethylations of reduced cystein side chains are routinely used when standard protocols for the enzymatic degradation of intact proteins are applied, these processes have to be taken into account when ECD spectra of the resulting peptides are evaluated.

FT-ICR ECD fragmentation represents a highly efficient tool for identification of the type of amino acid modification and their topology in the analysis of complex peptide mixtures generated by proteomics-based strategies.

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