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Complementary Structural Information from a Tryptic *N*-Linked Glycopeptide via Electron Transfer Ion/Ion Reactions and Collision-Induced Dissociation

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Abstract

Glycosylation is an important post-translational modification. Analysis of glycopeptides is difficult using collision-induced dissociation, as it typically yields only information about the glycan structure, without any peptide sequence information. We demonstrate here how a 3D-quadrupole ion trap, using the complementary techniques of collision induced dissociation (CID) and electron-transfer dissociation (ETD), can be used to elucidate the glycan structure and peptide sequence of the *N*-glycosylated peptide from a fractionated tryptic digest of the lectin from the coral tree, *Erythrina cristagalli*. CID experiments on the multiply protonated glycopeptide ions yield, almost exclusively, cleavage at glycosidic bonds, with little peptide backbone fragmentation. ETD reactions of the triply charged glycopeptide cations with either sulfur dioxide or nitrobenzene anions yield cleavage of the peptide backbone with no loss of the glycan structure. These results show that a 3D-quadrupole ion trap can be used to provide glycopeptide amino acid sequence information as well as information about the glycan structure.

Keywords

glycopeptide; tandem mass spectrometry; ion/ion reaction; electron-transfer dissociation

Introduction

Characterization of protein post-translational modifications, such as phosphorylation, disulfide bond formation, and glycosylation, is a key objective in proteomics. Glycosylation is likely the most common post-translational modification, and it has been suggested that glycoproteins comprise more than half of the proteins present in a eukaryotic cell.¹ Glycosylation affects protein solubility, cell to cell recognition, and biological activity. Mass spectrometry is a particularly attractive technique for the study of glycosylated proteins, due both to its relatively high sensitivity² and the relatively high precision and accuracy with which it can determine the masses of glycopeptides and glycoproteins. Tandem mass spectrometry is also a potentially powerful tool due to its ability to derive structural information about glycans and peptides as well as to localize sites of glycosylation.

A common approach to glycopeptide analysis is to enzymatically digest a glycoprotein into peptides and separate the mixture by reverse-phase liquid chromatography. A variety of mass spectrometers have been used for glycopeptide analysis, including quadrupole ion trap,^{3,4} triple quadrupole,^{5–7} quadrupole time-of-flight (Q-TOF),⁸ tandem time-of-flight (TOF-TOF),^{9,10} and Fourier transform ion cyclotron resonance (FT-ICR)^{11–13} instruments. Ion activation techniques that involve excitation of protonated or multiply protonated peptides,

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including collision-induced dissociation (CID),^{3–10} infrared multiphoton dissociation (IRMPD),^{12,14} and post-source decay (PSD),¹⁵ have been employed to elucidate the glycan structure, as well as the peptide sequence. These dissociation methods often lead to preferential cleavage of the glycosidic bonds rather than the polypeptide bonds and, as a result, usually provide information primarily about the glycan structure and not the peptide sequence. One solution to this problem is to cleave the glycan structure from the peptide using a glycosidic enzyme, allowing the analysis of each component separately.¹⁶ The disadvantage of this technique is that direct information about the site of glycosylation is lost.

An alternative dissociation strategy for glycopeptide analysis is to use electron capture dissociation (ECD).^{17,18} This process usually involves the conversion of a multiply protonated species to a hypervalent radical species one charge state lower. Although ECD of chitooligosaccharides has been reported to cleave glycosidic bonds,¹⁹ ECD of glycopeptides has been shown to preferentially cleave the peptide backbone while leaving glycan structures intact.^{12,13} This approach has been used successfully to localize sites of *N*-linked^{12,14} and *O*-linked^{8,13} glycosylation in proteins. Håkansson et al. have reported the use of ECD in conjunction with IRMPD to provide information about both the structure of the sugar and the peptide, as the two activation methods provide complementary information.^{12,14} While efficient ECD is currently performed only with FT-ICR instruments, it is desirable to be able to access the structural information afforded by ECD with other more widely available forms of tandem mass spectrometry. Some preliminary studies have been performed on adapting electrodynamic ion traps for ECD.^{20,21} Electron transfer ion-ion reaction chemistry has recently been shown to produce ECD-like results in linear^{22,23} and 3D^{24,25} electrodynamic ion trap instruments. In this work, we demonstrate that dissociation resulting from ion/ion electron transfer (ETD) in a 3D quadrupole ion trap, in combination with CID of the multiply protonated peptide, can be used to provide information about both peptide and glycan structures for a glycopeptide from the lectin of *E. cristagalli*.

Experimental Section

Methanol, acetonitrile, and glacial acetic acid were purchased from Mallinckrodt (Phillipsburg, NJ). Lectin from *Erythrina cristagalli* (Cockspur Coral Tree), TPCK treated trypsin, ammonium bicarbonate, nitrobenzene, and perfluoro-1,3-dimethylcyclohexane (PDCH) were obtained from Sigma-Aldrich (St. Louis, MO). Sulfur dioxide was purchased from Scott Specialty Gases (Troy, MI). Trifluoroacetic acid was obtained from Pierce (Rockford, IL).

The *E. cristagalli* lectin (0.75 mg) was dissolved in 0.5 mL of water containing 200 mM ammonium bicarbonate. TPCK treated trypsin (5 μ L of a 1 mg/mL aqueous solution) was added to the lectin solution to accomplish digestion. The solution was incubated at 38 °C for 3 h. After digestion, the resulting peptide mixture was separated by reversed-phase HPLC using an Aquapore RP-300 (7 μ m pore size, 100 $\times$ 4.6 mm i.d.) column (Perkin-Elmer, Wellesley, MA) operated at 1 mL/min. A linear 60 min gradient from Buffer A (0.1% TFA in water) to B (60:40 acetonitrile:water containing 0.09% TFA) was used for the separation. The fractions were lyophilized, and then dissolved in 50:49:1 methanol/water/acetic acid to yield a concentration of approximately 10 μ M prior to introduction to the mass spectrometer by nano-electrospray^{26,27} ionization.

Experiments were performed on a Hitachi (San Jose, CA) M-8000 quadrupole ion trap mass spectrometer that has been modified for ion/ion reactions, which has been described in detail previously.²⁸ Anions of PDCH, sulfur dioxide, and nitrobenzene, for either proton transfer or electron-transfer ion/ion reactions, were generated using atmospheric sampling glow discharge ionization (ASGDI).²⁹ The anions were introduced into the mass spectrometer through a hole in the ring electrode. Headspace vapors of PDCH and nitrobenzene were admitted into the

glow discharge source at pressures of 850 mTorr and 870 mTorr respectively, while sulfur dioxide gas was admitted to a pressure of 530 mTorr. A software TTL trigger connected to a fast high voltage pulser (GRX-1.5K-E, Directed Energy Inc., Fort Collins, CO) was used to pulse the discharge. Peptide cations were formed using nano-electrospray with the samples loaded into nanospray emitters pulled from borosilicate capillaries (1.5 mm o.d., 0.86 mm i.d.) using a P-87 Flaming/Brown micropipet puller (Sutter Instruments, Novato, CA). A stainless steel wire was inserted into the back of the capillary and 1.5–2 kV was applied to the wire for ionization.

A typical experiment consisted of about 1 s of cation injection time. This was followed by an isolation step using the Hitachi's filtered noise field (FNF)^{30,31} waveforms and by raising the amplitude of the radio frequency signal applied to the ring electrode of the ion trap to eject unwanted ions (~50 ms). For some experiments, SO_2^- anions were injected for 200–300 ms allowing for ion/ion reactions during that time, and an AC signal was applied to the endcaps of the ion trap to eject any SO_3^- ions (formed from SO_2^- via ion/molecule reactions in both the source and the ion trap). Due to the low RF levels required to trap SO_2^- ions, continual injection of anions was necessary during the reaction time to efficiently trap high mass positive ions (a process known as "trapping by proxy").³² For other experiments, anions derived from ASGDI of nitrobenzene headspace vapors were injected for approximately 100 ms into the ion trap. During this time a FNF waveform was applied to eject anions other than nitrobenzene $[\text{M}-\text{H}]^-$ and M^- ions. This injection period was followed by approximately 200 ms to allow for ion/ion reactions to occur. For both the SO_2 and nitrobenzene experiments, the remaining anions were ejected after the ion/ion reactions by raising the RF level of the trap, and cations were subsequently analyzed by resonance ejection. For some experiments, subsequent isolation and activation steps are performed prior to mass analysis. Isolation steps were performed with the FNF function, and an auxiliary Agilent (Palo Alto, CA) 33120A arbitrary waveform generator, controlled by a software TTL trigger, was used to resonantly excite ions of interest for approximately 300 ms. For one experiment, the charge state of the peptide cations were reduced using PDCH³³ to form predominately $[\text{M}+2\text{H}]^{2+}$ ions. These ions were then isolated and subjected to CID as described above. Spectra shown here are averaged over approximately 3–10 min (~30–150 scans).

Results

The *N*-glycosylated peptide used in the experiment produces triply charged ions when ionized by nano-electrospray. The average mass of the glycopeptide is 3002.13 Da. The glycan structure for the *E. cristagalli* lectin is a known Xylose type glycan consisting of the following structure, $\text{Man}\alpha 3(\text{Man}\alpha 6)-(\text{Xyl}\beta 2)\text{Man}\beta 4\text{GlcNAc}\beta 4(\text{Fuc}\alpha 3)\text{GlcNAc}$.³⁴ The peptide sequence and glycan structure are shown in Figure 1a. This particular glycopeptide was also studied with IRMPD and ECD in a FT-ICR instrument and the IRMPD data were consistent with this glycan structure.³⁵ The collision induced dissociation spectrum for the triply charged glycopeptide ion is shown in Figure 1a. The observed fragmentation is rich in information about the glycan structure, as the fragments correspond to cleavage of glycosidic bonds. However, there is no fragmentation of the peptide backbone. The entire structure of the glycan can be inferred from the CID spectrum, although the exact linkage types and locations of individual sugars cannot be determined. The most abundant fragments in the CID spectrum correspond to losses of the outermost sugars (Fucose (Fuc), Mannose (Man), or Xylose (Xyl)). Also, fragments corresponding to losses of two of the outermost sugars are observed (FucXyl, ManXyl, and FucMan). A series of doubly charged peptide ions, each containing pieces of the glycan structure, allows the inference of this glycan structure. It is important to note that in this experiment the glycan structure is known, but in a case where the glycan structure is unknown, it is doubtful that the glycan structure and composition could be as easily determined. Problems would likely arise from the isobaric nature of the glycan components and from more highly

branched glycan structures. Even if the complete structure cannot be determined, compositional information may still be of value. The peaks that allow the determination of the glycan structure are labeled in Figure 1a. The presence of the doubly charged fragment ion corresponding to the charged loss of XylMan₃-GlcNAc verifies the location of the fucose residue on the *N*-acetylglucosamine (GlcNAc) residue that is bound to the asparagine residue in the peptide. The CID results shown here are very similar to the IRMPD results obtained by Håkansson et al. in a FT-ICR, both in the appearance of the various cleavages of glycosidic bonds as well as the absence of peptide bond cleavages.³⁵

The doubly charged glycopeptide ion was formed via ion/ion proton transfer reactions with anions derived from glow discharge ionization of PDCH. The CID spectrum of the doubly charged ion is shown in Figure 1b. The observed fragmentation is not as rich in information as that observed in the spectrum of the triply protonated species, but information about the glycan structure is still present. The most abundant cleavages also result from the losses of the outermost sugar residues (Fuc, Man, Xyl) as neutral species. Neutral loss of two sugar residues is also observed, but the abundance is lower. Absent from the spectrum is the loss (either charged or neutral) of three of the outermost sugar residues that were present in the triply charged spectrum. However, a doubly charged ion corresponding to the loss of four sugars (XylMan₃) was observed. Singly charged ions that confirm the locations of the fucose and the two *N*-acetylglucosamines are observed. Two fragments that appear to arise from peptide bond cleavages are also observed in the spectrum (y_4^+ and y_{15}^{2+}). Both of these ions are observed along with the loss of a small molecule (water or ammonia). The y_4^+ ion localizes the glycan structure to the last 4 residues, but additional fragmentation would be needed to positively identify the location.

An alternative fragmentation method is to use ion/ion electron transfer reactions. In these experiments, the triply charged glycopeptide is subjected to ion/ion reactions with molecular anions of either sulfur dioxide or nitrobenzene. The post-ion/ion reaction spectrum of the glycopeptide with sulfur dioxide anions is shown in Figure 2a. The major products from the ion/ion reaction were doubly and singly charged parent glycopeptide ions. These signals are likely composed of a mixture of ions formed by proton transfer (major product) and electron transfer without dissociation. Ion/ion proton transfer is not expected to give rise to dissociation, whereas some fraction of the electron-transfer products is expected to dissociate.²⁵ The isotope peaks are not resolved, preventing the determination of the relative contributions of these products. In comparing the CID data with the ETD results, in terms of fragmentation behavior, the CID data of $[M+2H]^{2+}$ are most relevant since ETD of $[M+3H]^{3+}$ proceeds through $[M+3H]^{2+}$. In sharp contrast with the spectrum of Figure 1b, the spectrum in Figure 2a shows a series of singly charged z-type ions formed by electron-transfer dissociation of the peptide backbone. It is expected that the ions formed are z^{+} ions, but the mass accuracy and resolution of the instrument used for these experiments does not allow for differentiation between z^+ and z^{+} ions. The observed z-type ions form a contiguous series (z_4^+ to z_{14}^+) allowing the formulation of a peptide sequence tag that could be used to identify the protein if it were unknown. The z_1^+ to z_3^+ ions may have been formed but fall outside of the m/z range of the scan. The z_{15}^+ ion is absent from the spectrum, as it involves cleavage n-terminal to proline which would require cleavage of two bonds.¹⁷ The spectrum also contains some fragments corresponding to the loss of pieces of the glycan structure. These ions appear to arise from fragmentation of the undissociated triply and doubly charged ions during resonance ejection, as they are not present when the low mass cutoff is increased prior to mass analysis to eject the triply and doubly charged ions (data not shown). Noticeably absent in the spectrum are the complementary c-type ions, which are normally produced by both ECD and ETD. The structural information derived from this spectrum is very similar to that derived via ECD of this glycopeptide as well as a homologous glycopeptide derived from *E. corallodendron*.^{12, 35} However, it is interesting to note that the ECD work reported mostly c-type ions and

relatively few z-type ions. It is unclear whether this difference is characteristic of the chosen glycopeptide or a more general difference between ECD and ETD. Further work is warranted to determine the extent to which ECD (in the low pressure environment of the FT-ICR) and ETD (in an electrodynamic ion trap) are analogous. However, this difference suggests that the two approaches, while apparently similar in some respects, do not lead to identical products.

The triply charged glycopeptide ion was also subjected to electron-transfer dissociation reactions with the nitrobenzene $[M-H]^-$ and $M^{\cdot-}$ anions. The resulting spectrum is shown in Figure 2b. Again, the major reaction products correspond to the singly and doubly charged ions formed by both proton transfer and electron transfer with no dissociation, as was also noted for sulfur dioxide anions. The electron transfer dissociation fragments observed after reaction with nitrobenzene anions generally match those observed in the reaction with $SO_2^{\cdot-}$. Major differences include the absence of the z_{14}^+ ion and the observation of the c_{14}^+ , c_{15}^+ , and c_{16}^+ ions. Fragments corresponding to the loss of pieces of the glycan residue are also present. These fragments are the same as those observed in the $SO_2^{\cdot-}$ reaction data and presumably arise from CID of the doubly and triply charged parent species during resonance ejection (see above). An interesting observation in the post-ion/ion reaction spectrum with nitrobenzene anions is the appearance of a satellite peak 31 Da higher in mass with each of the z ions. These peaks are unlabeled in the spectrum, but are present for every observed z ion. They presumably result from a reaction between the polypeptide cation and the anion but the detailed origin of the 31 Da adduct is currently under further study. In any case, the appearance of the adduct ions may prove to be useful in distinguishing between z-type ions and c-type ions if it is observed on a consistent basis with other polypeptide ions. In Figure 2a,b, the z_8^+ ion has a mass similar to (within 1 Da) a fragment that could be formed from the loss of a piece of the glycan residue. The nitrobenzene anion reaction spectrum contains the z_8^+ ion with the 31 Da adduct, suggesting that the assignment as a z_8^+ product is likely to be correct. As with the $SO_2^{\cdot-}$ reaction data, the observed z ions form a contiguous series that could be used to construct a peptide sequence tag for protein identification. These newly observed c ions (c_{14}^+ to c_{16}^+), combined with the z ions, definitively localize the glycan structure to Asn₁₄ in the peptide.

Conclusion

A tryptic glycopeptide has been studied in a 3D-ion trap mass spectrometer by collision induced dissociation and electron transfer ion/ion reactions. CID experiments on the triply charged glycopeptide ion show cleavage at every glycosidic bond, without any cleavage of the peptide backbone. CID of the doubly charged glycopeptide ion, formed by ion/ion proton transfer reactions, shows cleavage at every glycosidic bond along with minimal cleavage of the peptide backbone. Ion/ion reactions between glycopeptide cations and anions of sulfur dioxide or nitrobenzene result in electron transfer with and without dissociation, and proton transfer. The electron transfer dissociation products of the glycopeptide show cleavage of the peptide backbone producing c- and z-type ions. Fragmentation was observed at almost every amino acid residue, but no cleavage of the glycosidic bonds was observed as a product of the ion/ion reactions. These results show that electron transfer dissociation can be used to provide amino acid sequence information for glycopeptides while collision induced dissociation provides information on the glycan structure. As both dissociation techniques can be readily effected in electrodynamic ion traps, these results show that this type of complementary information, previously only available on an FT-ICR instrument, is obtainable using a 3D-quadrupole ion trap.

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References

1. Apweiler R, Hermjakob H, Sharon N. *Biochim Biophys Acta* 1999;1473:4–8. [PubMed: 10580125]
2. Harvey DJ. *Proteomics* 2001;1:311–328. [PubMed: 11680878]
3. Demelbauer UM, Zehl M, Plematl A, Allmaier G, Rizzi A. *Rapid Commun Mass Spectrom* 2004;18:1575–1582. [PubMed: 15282782]
4. Wada Y, Tajiri M, Yoshida S. *Anal Chem* 2004;76:6560–6565. [PubMed: 15538777]
5. Carr SA, Huddleston MJ, Bean MF. *Protein Sci* 1993;2:183–196. [PubMed: 7680267]
6. Conboy JJ, Henion JD. *J Am Soc Mass Spectrom* 1992;3:804–814.
7. Huddleston MJ, Bean MF, Carr SA. *Anal Chem* 1993;65:877–884. [PubMed: 8470819]
8. Nemeth JF, Hochensang GP Jr, Marnett LJ, Caprioli RM. *Biochemistry* 2001;40:3109–3116. [PubMed: 11258925]
9. Wuhler M, Hokke CH, Deelder AM. *Rapid Commun Mass Spectrom* 2004;18:1741–1748. [PubMed: 15282773]
10. Kuroguchi M, Nishimura SI. *Anal Chem* 2004;76:6097–6101. [PubMed: 15481958]
11. Haselmann KF, Budnik BA, Olsen JV, Nielsen ML, Reis CA, Clausen H, Johnsen AH, Zubarev RA. *Anal Chem* 2001;73:2998–3005. [PubMed: 11467546]
12. Håkansson K, Cooper HJ, Emmett MR, Costello CE, Marshall AG, Nilsson CL. *Anal Chem* 2001;73:4530–4536. [PubMed: 11575803]
13. Mirgorodskaya E, Roepstorff P, Zubarev RA. *Anal Chem* 1999;71:4431–4436. [PubMed: 10546526]
14. Håkansson K, Chalmers MJ, Quinn JP, McFarland MA, Hendrickson CL, Marshall AG. *Anal Chem* 2003;75:3256–3262. [PubMed: 12964777]
15. Goletz S, Thiede B, Hanisch FG, Schultz M, Peter-Katalinic J, Muller S, Seiz I, Karsten U. *Glycobiology* 1997;7:881–896. [PubMed: 9363430]
16. Mechref Y, Novotny MV. *Chem Rev* 2002;102:321–369. [PubMed: 11841246]
17. Zubarev RA. *Mass Spectrom Rev* 2003;22:57–77. [PubMed: 12768604]
18. Zubarev RA, Kelleher NL, McLafferty FW. *J Am Chem Soc* 1998;120:3265–3266.
19. Budnik BA, Haselmann KF, Elkin YN, Gorbach VI, Zubarev RA. *Anal Chem* 2003;75:5994–6001. [PubMed: 14588042]
20. Silivra OA, Kjeldsen F, Ivonin IA, Zubarev RA. *J Am Soc Mass Spectrom* 2005;16:22–27. [PubMed: 15653360]
21. Baba T, Hashimoto Y, Hasegawa H, Hirabayashi A, Waki I. *Anal Chem* 2004;76:4263–4266. [PubMed: 15283558]
22. Syka JEP, Coon JJ, Schroeder MJ, Shabanowitz J, Hunt DF. *Proc Natl Acad Sci USA* 2004;101:9528–9533. [PubMed: 15210983]
23. Coon JJ, Syka JEP, Schwartz JC, Shabanowitz J, Hunt DF. *Int J Mass Spectrom* 2004;236:33–42.
24. Chrisman, P. A.; Pitteri, S. J.; Hogan, J. M.; McLuckey, S. A. *J. Am. Soc. Mass Spectrom.* in press.
25. Pitteri, S. J.; Chrisman, P. A.; Hogan, J. M.; McLuckey, S. A. *Anal. Chem.* 2005, published ASAP.
26. Kelleher NL, Senko MW, Siegel MM, McLafferty FW. *J Am Soc Mass Spectrom* 1997;8:380–383.
27. Van Berkel GJ, Asano KG, Schnier PD. *J Am Soc Mass Spectrom* 2001;12:853–862. [PubMed: 11444609]
28. Reid GE, Wells JM, Badman ER, McLuckey SA. *Int J Mass Spectrom* 2003;222:243–258.
29. McLuckey SA, Glish GL, Asano KG, Grant BC. *Anal Chem* 1988;60:2220–2227.
30. Goeringer DE, Asano KG, McLuckey SA, Hoekman D, Stiller SE. *Anal Chem* 1994;66:313–318.
31. Kelley, P. E. *Mass Spectrometry Method using Notch Filter*, U.S. Patent 5,134,286, July 28, 1992.
32. McLuckey SA, Wu J, Bundy JL, Stephenson JL Jr, Hurst GB. *Anal Chem* 2002;74:976–984. [PubMed: 11925000]
33. Stephenson JL Jr, McLuckey SA. *Int J Mass Spectrom Ion Process* 1997;162:89–106.
34. Ashford D, Dwek RA, Welply JK, Atamayakul S, Homans SW, Lis H, Taylor GN, Sharon N, Rademacher TW. *Eur J Biochem* 1987;166:311–320. [PubMed: 3609010]
35. Håkansson K, Cooper HJ, Hudgins RR, Nilsson CL. *Curr Org Chem* 2003;7:1503–1525.

Figure 1. Collision-induced dissociation spectrum of a) the $[M+3H]^{3+}$ glycopeptide ion, and b) the $[M+2H]^{2+}$ glycopeptide ion formed by ion/ion proton-transfer reactions with PDCH.

Figure 2.

Electron-transfer dissociation spectrum of a) the $[M+3H]^{3+}$ glycopeptide ion after reaction with sulfur dioxide anions, and b) the $[M+3H]^{3+}$ glycopeptide ion after reaction with nitrobenzene anions. Ions corresponding to fragmentation of the glycan structure likely arises from CID of the $[M+3H]^{3+}$ and $[M+2H]^{2+}$ ions during mass analysis, as described in the text.