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Construction of a Database of Collision Cross Section Values for Glycopeptides, Glycans, and Peptides Determined by IM-MS

Rebecca S. Glaskin[†], Kshitij Khatri, Qi Wang[‡], Joseph Zaia, and Catherine E. Costello^{*}

Center for Biomedical Mass Spectrometry, Department of Biochemistry, Boston University School of Medicine, Boston, Massachusetts 02118-2646, United States

Abstract

An ion mobility quadrupole time-of-flight mass spectrometer was used to examine the gas-phase structures of a set of glycopeptides resulting from proteolytic digestion of the well-characterized glycoproteins bovine ribonuclease B, human transferrin, bovine fetuin and human α_1 -acid glycoprotein, the corresponding deglycosylated peptides, and the glycans released by the endoglycosidase PNGase F. When closely related glycoforms did not occur naturally, exoglycosidases were used to achieve stepwise removal of individual saccharide units from the nonreducing termini of the multiantennary structures. Collision cross sections (CCS) were calculated and plotted as a function of mass-to-charge ratio. Linear trendlines were observed for the glycoforms of individual *N*-linked glycopeptides, the deglycosylated peptides, and the released, deuterio-reduced permethylated glycans. For the glycoforms of a given glycopeptide or set of derivatized glycans, the slope of the line connecting CCS values remained similar for the $[M + 3H]^{3+}$ ions observed as the glycan antennae were shortened by stepwise exoglycosidase treatments; this trend was consistent regardless of the peptide length or the saccharide removed. The results form the basis for a database of CCS values and the CCS increments that correspond to changes in glycoform compositions.

Graphical abstract

^{*}Corresponding Author: Address: Prof. Catherine E. Costello Center for Biomedical Mass Spectrometry Boston University School of Medicine 670 Albany St, room 511 Boston, MA 02118-2646. Tel.: (617) 638-6490. Fax: (617) 638-6761. cecmsms@bu.edu.

ORCID: Kshitij Khatri: 0000-0002-6592-869X

Joseph Zaia: 0000-0001-9497-8701

Catherine E. Costello: 0000-0003-1594-5122

[†]**Present Addresses:** Agilent Technologies, Inc., Wilmington, DE 19808

[‡]**Present Addresses:** Waters Corporation, Milford, MA 01757

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Interest in ion mobility spectrometry (IMS) has grown because of its capability to separate conformations of gas-phase ions, including biomolecules, explosives, drugs, and chemical-warfare agents.^{1,2} In IMS, ions are periodically introduced into a drift tube that consists of a chamber filled with a buffer gas that contains a series of electrostatic lenses to which a weak uniform electric field is applied. Ions separate in the drift tube based on their cross-section-to-charge ratios, as a result of the applied electric field and interactions with the buffer gas.^{1–5}

A major benefit of IMS is the time scale of the mobility separation, which enables the coupling of a drift tube to a time-of-flight mass spectrometer. With the IMS separation occurring on a millisecond time scale and flight times on a microsecond time scale, thousands of mass spectra can be collected during each ion mobility separation, generating a two-dimensional data array wherein the mobilities and mass-to-charge (m/z) values of ions are recorded.⁶ In addition to the extra dimension of separation produced by having IMS prior to mass spectrometry, structural information can be obtained with the calculation of collision cross section (CCS, Ω) values, which provide information on the orientationally averaged surface area of the ions.⁷

IMS has demonstrated its capability for separations of lipids, carbohydrates, and peptides, as well as proteins and protein assemblies.^{8–19} This capability results from differences in the CCS values that occur as the mass is increased, for each of these different classes of biomolecules. Previous work has shown that each class tends to fold into particular patterns, dependent on the charge state, degrees of freedom, and intramolecular folding forces. IMS also separates charge states into distinct trend lines, with ions falling along these lines exhibiting a strong dependence between mobility (CCS) and mass. When CCS is plotted as a function of m/z , more compact ions will fall below the trend line. This has been displayed previously for the series of biomolecules described above.¹¹ Although GlycoMob, a database for CCS of free glycans, was announced recently,²⁰ to date there have been few reports that can provide a database that includes glycopeptides, the corresponding released glycans and deglycosylated peptides. Such data can be used to determine whether trends in the CCS values of glycopeptides follow those determined for peptides or glycans and whether their variation shows some dependence on peptide length or glycan composition and branching.

Most eukaryotic proteins are modified by *N*- and/or *O*-linked glycosylation.^{21,22} Cell surface and extracellular matrix proteins may include one or more *N*-linked sites where the glycan is covalently bound to asparagine,^{23–26} almost always in a consensus sequon Asn-Xxx-Thr/Ser (Xxx = Pro).^{27–29} In addition, *O*-linked glycans can modify the hydroxyl groups of Ser/Thr and, less frequently, hydroxyPro.^{29,30} Glycosylation impacts the structure, folding, stability, function, and activity of proteins.^{23–26} In particular, glycosylation enables binding to modular lectin domains present on many extracellular proteins.^{31,32} Aberrant glycosylation associated with cancer, neurodegenerative disorders, and genetic abnormalities reflects disease physiology,^{33–35} and many infectious agents show specificity for particular cell surface glycans.³⁶ Thus, essential physiological functions including cell–cell and cell–matrix recognition,³⁷ cellular adhesion,³⁸ and inter- and intracellular interactions depend on protein–carbohydrate interactions.³⁸

Glycoconjugate glycan expression reflects enzyme-dependent remodeling and biosynthetic steps in the Golgi apparatus and endoplasmic reticulum that vary according to cell type, development, disease state, and species-specific expression of glycosyltransferases.^{39,40} In addition, the population of glycans present at each site reflects the efficiencies of the individual biosynthetic steps as that particular glycoprotein is generated and processed. Thus, a given site exists as a set of glycoform variants that differ with regard to degree of occupancy, composition, linkage positions, and branching points.⁴¹ Each glycoprotein is expressed as a set of glycoforms that share a common amino acid sequence as the backbone but differ in the localizations, structures, or sequences of carbohydrate units.³⁷

Owing to the large number of possible glycan structures and localizations, glycoprotein and even glycopeptide compositions cannot be assigned solely from high resolution mass measurements.⁴² Tandem MS experiments increase the structural detail for glycopeptides; however, statistically rigorous scoring methods for glycopeptide MS/MS data are not yet available. Thus, the confidence level for many glycoproteomics experiments remains poorly defined. IMS offers the advantages of providing an additional, orthogonal mode for resolving mixture components and CCS data that may be used as a feature to improve the depth to which glycopeptides may be reliably assigned, provided that there is an understanding of the influence of the glycan structure on mobility.⁴²

Here, we have measured the mobilities of glycopeptides produced by protease digestions of well-characterized glycoproteins and have utilized exoglycosidases to generate additional glycoforms, in order to observe how the volume increments that are due to individual monosaccharide units affect the CCS values. We are compiling the measured values into a database that we are developing for *N*-linked glycopeptides, reduced and permethylated *N*-glycans, and deglycosylated/deamidated peptides. In particular, we observed that the cross section values measured for the $[M+3H]^{3+}$ ions of a given glycopeptide or glycan changed linearly as the number of residues of a particular monosaccharide at the termini of its antennae was varied and that the incremental differences were dependent on the identity of the mono-saccharide. Furthermore, the relationships showed little dependence on the peptide structures. This work establishes the feasibility of building a database of glycopeptide and glycan mobilities as a function of glycan composition that will be useful for mobility-based

glycopeptide and glycan profiling, which will likely extend to other types of glycoconjugates as well.

Experimental Section

Glycoproteins, Enzymes, Solvents, and Reagents

Bovine ribonuclease B (RNase B), human transferrin, bovine fetuin, and human α_1 -acid glycoprotein (AGP) were obtained from Sigma-Aldrich (St. Louis, MO). Sequencing grade trypsin and chymotrypsin were purchased from Promega Corp. (Madison, WI). Endoglycosidase F and exoglycosidases were prepared by New England Biolabs (Beverly, MA). LC-MS solvents were acquired from Fisher Scientific (Pittsburgh, PA). Unless specifically stated, all other reagents were obtained from Sigma-Aldrich.

Methods

Glycoprotein Reduction, Alkylation, and Protease Digestion

Each glycoprotein was reduced, alkylated, and subjected to tryptic or chymotryptic digestion, as described (Supporting Information). The glycopeptide fraction was enriched with a ProteoExtract Glycopeptide Enrichment Kit (EMD Millipore, Billerica, MA), following the manufacturer's protocol.

Glycopeptide Sample Preparation, Stepwise Exoglycosidase Digestion, and Endoglycosidase Release of *N*-Linked Glycans (see further details in the Supporting Information)

A 20- μ g aliquot of each glycopeptide-enriched fraction was reconstituted with 80:20:0.1 water/acetonitrile/formic acid ($\text{H}_2\text{O}/\text{ACN}/\text{FA}$) to a concentration of 2 $\mu\text{g}/\mu\text{L}$; these solutions were diluted (with 99:1:0.1 $\text{H}_2\text{O}/\text{ACN}/\text{FA}$) to 200 ng/ μL prior to C18-reversed phase separation.

Another portion of each glycopeptide-enriched fraction (20–40 μg) was sequentially treated with three exoglycosidases: α 2-3,6,8-neuraminidase, β 1,4-galactosidase, and β -*N*-acetylglucosaminidase. The products from each step were desalted using Pierce C18 spin columns (Thermo Scientific, Rockford, IL) and prepared for analysis by LC-IM-MS in 80:20:0.1 $\text{H}_2\text{O}/\text{ACN}/\text{FA}$.

N-glycans were released from glycopeptides with PNGase F using the protocol described in the Supporting Information. The peptides resulting from the PNGase F release were resuspended in 25 μL of 90:10:0.1 $\text{H}_2\text{O}/\text{ACN}/\text{FA}$. For LC-MS, 0.5 μL of each peptide solution was diluted into 4.5 μL of a mixture of 99:1:0.1 $\text{H}_2\text{O}/\text{ACN}/\text{FA}$; 2.5 μL of the resulting solution was injected by the autosampler.

Glycan Reduction, Permethylation, and Analysis

The glycan pools resulting from endoglycosidase F treatments of RNase B, transferrin, fetuin, and AGP, with or without subsequent stepwise exoglycosidase treatment, were each reduced with NaBD₄ and then permethylated with a method introduced by Ciucanu and

Kerek⁴³ and later modified by Ciucanu and Costello,⁴⁴ as described in the Supporting Information. The derivatized glycan products were suspended in 50 μL of 50:50:0.1 $\text{H}_2\text{O}/\text{ACN}/\text{FA}$. For HPLC-MS analysis 1 μL of the RNase B glycans solution was diluted 1:8 with 90:10:0.1 $\text{H}_2\text{O}/\text{ACN}/\text{FA}$; for transferrin, fetuin, and AGP, the glycan solution was diluted 1:4 with 96:4:0.1 $\text{H}_2\text{O}/\text{ACN}/\text{FA}$. For each experiment, 8 μL was injected.

Instrumentation

The Agilent 1200 series HPLC-Chip Cube interface equipped with a Polaris-HR-Chip-3C18 LC chip was coupled to a 6560 Ion Mobility Quadrupole Time-of-Flight (IM-QTOF) mass spectrometer (Agilent Technologies, Santa Clara, CA). The LC system included an autosampler, a binary capillary-flow pump used for loading samples, and a nanoflow gradient pump. Tryptic and chymotryptic digests of each reduced and alkylated protein were separated using the Polaris-HR-Chip-3C18 (3 μm , 180 Å pore size) that had a 360 nL enrichment column followed by a 75 $\mu\text{m} \times 150$ mm separation/analytical column. Further details on the HPLC separation conditions are in the Supporting Information. The HPLC chip was held at a DC bias of 1950–2250 V above the ion source entrance aperture, which is followed by a dual ion funnel interface into the 6560 IM-QTOF analyzer. IMS separation took place in the ~80-cm-long drift tube, operated at 18.5 V/cm (temperature, 30.5–33.5 °C; pressure, 3.91 to 4.04 Torr nitrogen). Ions exiting the drift tube are refocused in a rear ion funnel prior to entering the mass analyzer. The dual ion funnel interface and rear ion funnels were operated at 100 and 120 V peak-to-peak, respectively. For the Agilent tune mix, the typical values were as follows: drift resolution, 50; mass resolution, 30 000; mass error, 2 ppm. These values are consistent with the manufacturer's specifications for drift and mass resolving power and mass accuracy. As discussed below, likely due to linkage and conformational heterogeneity, the drift peak widths recorded during glycopeptide and glycan sample measurements were wider, and consequently the observed drift resolution was lower, 15–60, whereas the mass resolution and measurement errors were still within the usual limits: mass resolution, 24 000–32 000; mass error, 1–10 ppm. The tune mix was infused during LC-MS runs, and mass calibration was corrected based on its signal at m/z 1221.

LC-IM-MS data files were extracted and deconvoluted using DeconTools.⁴⁵ Our software package GlycReSoft⁴⁶ was used to match the deconvoluted peak lists against theoretical peptide and glycopeptide neutral mass lists, with a mass tolerance of 20 ppm.

All measurements were made in triplicate. A representative plot of m/z vs flight time is shown in Figure 1. Measured CCS values were plotted vs m/z . Linear fits were applied, unless noted otherwise. The results are presented in Figures 2–6 and Supporting Information Figures S1–S13.

Results and Discussion

Analysis of RNase B Glycopeptides

Previous reports on IM-MS of the high mannose (Man) glycans of RNase B have analyzed the glycans released after tryptic digestion of the glycoprotein.^{47–49} The Clemmer group, utilizing a 1-m drift tube coupled to a linear ion trap, detected four isomeric structures for

doubly sodiated Man₇.⁴⁷ The Bowers and Harvey groups used a home-built drift tube and a Synapt G2-S instrument (Waters Corporation, Manchester, U.K.).⁴⁸ Harvey later used both unmodified and modified Synapt HDMS instruments (Waters Corporation, Manchester, U.K.) to analyze these glycans.⁴⁹ Their experiments distinguished the high Man *N*-glycan isomers based on drift time and determined their absolute CCS values. IM-MS studies of the proteolytic glycopeptides obtained from the proteins analyzed here (including RNase B) have not been reported, nor have the IM-MS properties been determined for the shortened glycoforms of the peptides or for the released, reduced, and permethylated glycans.

In our experiments, we first studied the RNase B tryptic glycopeptide ³⁴N*LTK³⁷ (where N* = occupied glycosylation site). Figure 1 displays a 2-D plot of *m/z* vs drift time over the drift time range during which the Man₅₋₉ [M+2H]²⁺ ions were observed. These results are comparable to those obtained previously by Bowers and Harvey for the released glycans.

For the Man₅₋₉ glycoforms observed in Figure 1, the extracted drift time distributions are plotted in Figure 2A. The drift time distributions for the [M+2H]²⁺ ions display multiple peaks for Man₆₋₈, consistent with separation of the known multiple isomeric structures.

Here and elsewhere, the operating parameters were maintained the same for measurements on the standards and the glycopeptide samples, so it can be presumed that the instrument resolving power does not change between recording the drift times of standards and samples. Therefore, the observations of lower drift time resolution for the glycopeptides and glycans (in comparison to the data obtained for the polyfluoroalkoxyphosphazine standards and the deglycosylated peptides) and multiple peaks at given *m/z* values are likely due to the presence of multiple conformers of individual isomers, as well as branching isomers, some of which overlap in their drift times, as illustrated in Supporting Information Figure S14.

CCS values calculated from the drift times obtained for the [M+2H]²⁺ ions of the Man₅₋₉ glycoforms ranged from 400 to 500 Å² (Figure 2A, Table S1). The measured CCS values were plotted as a function of *m/z* (Figure 2B). Two distinct linear trends can be observed as the number of Man units on the glycopeptide varies, one corresponding to the compact conformations (peaks falling along the blue line in Figure 2A) observed for Man₅₋₉, and the other, to the elongated conformations (peaks falling along the red line in Figure 2A) of less highly branched glycoforms of Man₆₋₈.

Analysis of Transferrin Glycopeptides

Transferrin has two *N*-linked glycosylation sites, each carrying a complex biantennary structure.^{50,51} The transferrin glycopeptides were analyzed using procedures similar to those described above. However, unlike RNase B, abundant shorter glycoforms of transferrin do not occur naturally (except in the case of disease or metabolic disorders). We therefore used exoglycosidases to sequentially remove individual saccharide units from the termini of the biantennary structures, in order to produce convenient quantities of the shorter glycoforms. Measured CCS values are listed in Table S1.

Figure 3 displays the results obtained for tryptic glycopeptides corresponding to the two known glycosylation sites of transferrin, after modification by carbamidomethylation (Cam)

at cysteine, $^{421}\text{C}(\text{Cam})\text{GLVPVLAENYN}^*\text{K}^{433}$ and $^{622}\text{QQQHLFGSN}^*\text{VTDC}(\text{Cam})\text{SGNFC}(\text{Cam})\text{LFR}^{642}$. Figure 3A and D, B and E, and C and F display the CCS plotted vs m/z for the glycoforms that varied in their content of sialic acid (NeuAc), galactose (Gal), and *N*-acetylglucosamine (GlcNAc), in that order, after these residues were stepwise removed from the nonreducing termini.

In Figure 3A, the CCS values obtained for the $[\text{M}+4\text{H}]^{4+}$ and $[\text{M}+3\text{H}]^{3+}$ ions of the biantennary glycopeptide $^{421}\text{C}(\text{Cam})\text{GLVPVLAENYN}^*\text{K}^{433}$ are plotted vs m/z for the species containing NeuAc₀₋₂. Unique linear trendlines can clearly be distinguished for the two observed charge states, as a result of the increased force experienced by ions in the higher charge state during travel through the gas-filled drift tube. Linear trends were also observed for the biantennary glycoforms with Gal₀₋₂ (Figure 3B) and then GlcNAc₀₋₂ (Figure 3C) at the nonreducing termini.

Figure 3D shows plots of CCS vs m/z for the $[\text{M}+4\text{H}]^{4+}$ and $[\text{M}+3\text{H}]^{3+}$ ions observed for the species with NeuAc₀₋₂ on the biantennary structure of the glycopeptide at the second glycosylation site, $^{622}\text{QQQHLFGSN}^*\text{VTDC}(\text{Cam})\text{SGNFC}(\text{Cam})\text{LFR}^{642}$. Figure 3E presents the results obtained for the series Gal₀₋₂ resulting from subsequent β -galactosidase treatment of the desialylated glycoforms. Both compact and elongated conformations were observed for the $[\text{M}+4\text{H}]^{4+}$ and $[\text{M}+3\text{H}]^{3+}$ ions. Figure 3F shows that, after further treatment with β -*N*-acetylglucosaminidase, the $[\text{M}+3\text{H}]^{3+}$ ions of the GlcNAc₀₋₂ glycoforms also exhibited a linear trend.

A parallel set of IM-MS analyses was carried out after the digestion of transferrin with chymotrypsin, to determine if the changes in the observed CCS were primarily dependent on the glycan composition or were influenced by the length of the peptide. Figure S1 shows the CCS plotted as a function of m/z for the $[\text{M}+3\text{H}]^{3+}$ ions of the NeuAc₀₋₃ glycoforms of the glycopeptides that resulted from tryptic and chymotryptic digests of transferrin. The line slope corresponding to CCS vs m/z with NeuAc₀₋₂ was 0.28 for the tryptic glycopeptide $^{622}\text{QQQHLFGSN}^*\text{VTDC}(\text{Cam})\text{SGNFC}(\text{Cam})\text{LFR}^{642}$ and 0.27 for the chymotryptic glycopeptide $^{627}\text{FGSN}^*\text{VTDC}(\text{Cam})\text{SGNF}^{638}$. This demonstrated that the change in CCS for the $[\text{M}+3\text{H}]^{3+}$ ions corresponding to variation in the number of NeuAc residues on glycopeptides bearing the same glycosylation site and biantennary *N*-glycan was independent of the peptide length.

Analysis of Fetuin Glycopeptides

Complex *N*-glycans of bovine fetuin have been previously studied by MS.⁵² Here, we analyzed peptides corresponding to one of the three occupied glycosylation sites. The procedures for sequential exoglycosidase treatments followed those above. Measured CCS values are listed in Table S1.

In Figure S2, CCS was plotted vs m/z for full-length tryptic glycopeptides from bovine fetuin and their products after two stepwise glycosidase treatments, $^{145}\text{LC}(\text{Cam})\text{PDC}(\text{Cam})\text{-PLLAPLN}^*\text{DSR}^{159}$, and its analog that contains one missed cleavage site, $^{144}\text{KLC}(\text{Cam})\text{PDC}(\text{Cam})\text{PLLAPLN}^*\text{DSR}^{159}$. These exhibited similar trends. For each set, we observed $[\text{M}+5\text{H}]^{5+}$, compact $[\text{M}+4\text{H}]^{4+}$, elongated $[\text{M}+4\text{H}]^{4+}$, and $[\text{M}+3\text{H}]^{3+}$ ions.

Although the CCS vs m/z plots showed similar slopes for the $[M+3H]^{3+}$ ions as the number of mono-saccharide units varied, the $[M+4H]^{4+}$ ions did not exhibit a linear trend.

Similar experiments were performed on the fetuin triantennary glycopeptides. These trends followed those described above for the biantennary transferrin glycopeptides. Figure S2 shows plots of CCS as a function of m/z for the triantennary complex *N*-linked glycopeptides resulting from the tryptic digestion of fetuin after sequential treatments with α 2-3,6,8 neuraminidase, β 1,4-galactosidase, and β -*N*-acetylglucosaminidase.

Fetuin was also digested with chymotrypsin. The results obtained as the numbers of (A) NeuAc and (B) Gal residues varied for the triantennary chymotryptic glycopeptide $^{156}\text{N}^*\text{DSRVVHAVEVAL}^{168}$ are shown in Figure 4. These results exhibited no significant difference in the variance of CCS upon change in the number of NeuAc residues present on two glycopeptides containing the same sequon and the same inner glycan structure but composed of different numbers of amino acids, the tryptic peptide $^{145}\text{LC}(\text{cam})\text{PDC}(\text{cam})\text{-PLLAPLN}^*\text{DSR}^{159}$ and the chymotryptic peptide $^{156}\text{N}^*\text{DSRVVHAVEVAL}^{168}$. As the number of NeuAc residues changed, the two sets of glycoforms exhibited the same slope for the plot of CCS vs m/z for the triply charged species. For the $[M+4H]^{4+}$ ions, the slope of the line corresponding to variation in the number of NeuAc residues differed significantly for this pair of glycopeptides (0.33 vs 0.22; Figure 4A). It is likely that the excess charge on the $[M+4H]^{4+}$ ions increases the extension of the overall structure as compared to $[M+3H]^{3+}$ ions; the increased flexibility of the $[M+4H]^{4+}$ ions which have a more elongated structure due to increased Coulombic repulsion is also reflected by the difference in the slopes for the $[M+4H]^{4+}$ ions, whereas the slopes for the $[M+3H]^{3+}$ ions are almost identical. Figure 4B shows the incremental changes due to variation in the terminal Gal residues on the asialo glycoforms observed for $[M+3H]^{3+}$ ions of glycopeptides resulting from tryptic and chymotryptic digestions. Here again, the situation was reversed: the slopes observed for CCS vs m/z were tryptic glycopeptides, 0.26; chymotryptic, 0.31.

Figure S3 displays values measured for the NeuAc₀₋₃ series of the $[M+4H]^{4+}$ and $[M+3H]^{3+}$ ions of the triantennary glycopeptide $^{98}\text{AN}^*\text{C}(\text{Cam})\text{SVRQQTQHAVE}^{111}$. Figure S4 displays similar plots for the triantennary chymotryptic glycopeptide $^{98}\text{AN}^*\text{C}(\text{Cam})\text{SVRQQTQHAVEGDC}(\text{Cam})\text{-DIHVL}^{119}$, analyzed for sequential variances in the numbers of (A) NeuAc, (B) Gal, and (C) GlcNAc residues. For NeuAc₀₋₃, $[M+4H]^{4+}$ and $[M+3H]^{3+}$ ions both displayed linear trends. The slope for the line for CCS vs m/z observed for the $[M+3H]^{3+}$ ions with NeuAc₀₋₃ was 0.26, the same value observed for the $[M+3H]^{3+}$ ions of $^{98}\text{AN}^*\text{C}(\text{Cam})\text{-SVRQQTQHAVE}^{111}$ with NeuAc₀₋₃ (Figure S3). A linear trend was determined for the $[M+4H]^{4+}$ and $[M+3H]^{3+}$ ions for Gal₀₋₃ and for $[M+3H]^{3+}$ ions with GlcNAc₀₋₃.

Figure S5 displays a plot of CCS vs m/z for variance in the numbers of (A) NeuAc, (B) Gal, and (C) GlcNAc residues on the triantennary glycoforms of the fetuin chymotryptic glycopeptide $^{172}\text{NAESN}^*\text{GSYL}^{180}$.

Analysis of α 1-Acid Glycoprotein Glycopeptides

Parallel analyses were carried out for human AGP, a glycoprotein that contains five *N*-linked glycosylation sites.⁵³ For AGP, tryptic glycopeptides exhibited zero, one, or two missed cleavage sites. Both tetra-antennary and triantennary structures were observed for most of the AGP glycopeptides discussed here. Measured CCS values are listed in Table S1.

For the glycoforms of the AGP tryptic glycopeptide ⁸⁷QNQC(Cam)FYN*SSYLVNQR¹⁰¹ differing in the numbers of (A) NeuAc, (B) Gal, and (C) GlcNAc residues, CCS was plotted vs *m/z*, for Figure S6. Figure S6A displays the glycoforms containing NeuAc₀₋₄ observed as [M+5H]⁵⁺ ions and clearly distinguished from the [M+4H]⁴⁺ ions.

Both tetra-antennary and triantennary structures for the [M+4H]⁴⁺ ions fall along the same diagonal with similar slopes, 0.35 and 0.37, respectively. For the [M+4H]⁴⁺ ions of tetra-antennary and triantennary glycoforms with Gal₀₋₄, compact and elongated structures were observed, with comparable trendlines (Figure S6B). The values determined for the [M+3H]³⁺ ions for the structures shown in Figure S6B overlap along the same diagonal and display linear trends for the plots of CCS vs *m/z*, with slopes of 0.29 (tetra-antennary structures) and 0.27 (triantennary). Figure S6C displays a plot of CCS vs *m/z* with a linear trendline applied for the [M+3H]³⁺ ion series corresponding to tetra-antennary structures with GlcNAc₀₋₄.

Both tetra- and triantennary structures were observed for glycoforms of the AGP peptide ⁹⁸NVQREN*GTISRY¹⁰⁹. Linear trends were found for CCS plotted vs *m/z* (Figure 5A); the observed slopes were tetra-antennary [M+4H]⁴⁺ ions, 0.37; triantennary [M+4H]⁴⁺, 0.40; tetra-antennary [M+3H]³⁺, 0.26; and triantennary [M+3H]³⁺, 0.30.

In Figure 5B, CCS was plotted vs *m/z* for the series NeuAc₀₋₃ on the triantennary structures of [M+3H]³⁺ ions of the tryptic and chymotryptic AGP glycopeptides ¹⁰²EN*G-TISR¹⁰⁸ and ⁹⁸NVQREN*GTISRY¹⁰⁹. The slopes for CCS vs *m/z* were tryptic glycopeptides, 0.29; chymotryptic, 0.30. As discussed above for fetuin, the observed changes in CCS were similar for the pair of glycopeptides with the same sugar attached, independent of the difference in peptide lengths. Here again, it appeared that the slope in the correlation of CCS with incremental changes in glycan depended on sugar composition and was independent of peptide length.

Analyses similar to those described above were conducted on additional glycopeptides resulting from tryptic and chymotryptic digestion of AGP and sequential treatment with the exoglycosidases; plots based on these results are presented in the Supporting Information.

Figure S7A displays the CCS plotted as a function of *m/z* for [M+4H]⁴⁺ and [M+3H]³⁺ ions observed for tetra- and triantennary structures of the AGP glycopeptide ¹⁰²EN*G-TISR¹⁰⁸. The rates of CCS changes for the [M+4H]⁴⁺ ions, for increments of NeuAc residues, were similar. Especially for the [M+3H]³⁺ ions, variation in the number of saccharide units in the multiantennary structures had less impact on the slope of CCS vs *m/z* than was observed for the biantennary structures.

For a tryptic AGP glycopeptide resulting from a missed cleavage, $^{102}\text{EN}^*\text{GTISRYVGGQEHLILR}^{123}$, the $[\text{M}+5\text{H}]^{5+}$ and $[\text{M}+4\text{H}]^{4+}$ ions both showed linear trends for CCS vs m/z (Figure S7B). The line slopes observed for the $[\text{M}+5\text{H}]^{5+}$ ions for two multiantennary structures deviated from one another (triantennary, 0.23; tetra-antennary, 0.32), whereas the slopes for the $[\text{M}+4\text{H}]^{4+}$ ions were similar (triantennary, 0.34; tetra-antennary, 0.29). Here again, as the charge state increases, the structures would become more elongated and have increased flexibility, perhaps allowing more interaction with the peptide. Figure S7C shows the results obtained for $[\text{M}+4\text{H}]^{4+}$ and $[\text{M}+3\text{H}]^{3+}$ ions for the triantennary glycopeptide, with variation in Gal.

For the tetra- and triantennary glycoforms of $^{87}\text{QDQC}-(\text{Cam})\text{IYN}^*\text{TTYLVNQR}^{101}$ with variance NeuAc (Figure S8A), the slopes resulting from the linear fits applied to the $[\text{M}+5\text{H}]^{5+}$ ions deviated more than those observed for the compact and extended forms of the $[\text{M}+4\text{H}]^{4+}$ and $[\text{M}+3\text{H}]^{3+}$ ions, suggesting that increased Coulombic repulsion allows the antennae to adopt more elongated and flexible structures. The values obtained for the $[\text{M}+3\text{H}]^{3+}$ ions overlapped along a comparable diagonal; the slopes in the plots of the CCS values for NeuAc₀₋₄ were tetra-antennary, 0.26; triantennary, 0.29.

For glycoforms with terminal Gal₀₋₄ on tetra- and triantennary structures of this glycopeptide, the compact and elongated conformations observed for $[\text{M}+4\text{H}]^{4+}$ ions of both sets of structures displayed linear trends, with deviations in their slopes (Figure S8B). The $[\text{M}+3\text{H}]^{3+}$ ions of these structures exhibited comparable diagonals (tetra-antennary slope, 0.20; triantennary, 0.17).

Results for the AGP tryptic glycopeptide $^{58}\text{SVQEIQATF-FYFTPN}^*\text{K}^{73}$ were similarly analyzed (Figure S8C); CCS values were plotted vs m/z for $[\text{M}+3\text{H}]^{3+}$ to $[\text{M}+5\text{H}]^{5+}$ ions. $[\text{M}+5\text{H}]^{5+}$ ions were observed for the tetra-antennary structure. CCS vs m/z plots for the $[\text{M}+4\text{H}]^{4+}$ ions fall along similar diagonals (tetra-antennary slope, 0.32; triantennary, 0.26) with variation in NeuAc. The $[\text{M}+3\text{H}]^{3+}$ ions exhibited similar behavior (tetra-antennary slope, 0.25; triantennary, 0.26).

For the triantennary glycoforms of the AGP tryptic glycopeptide $^{52}\text{NEEYN}^*\text{K}^{57}$ (NeuAc₀₋₃), both compact and elongated structures were observed for the $[\text{M}+4\text{H}]^{4+}$ ions (Figure S8D). Compact ions showed a quadratic trend for CCS vs m/z , whereas elongated ions followed a linear trend. Compact ions for this particular charge state may exhibit tighter folding for glycoforms having fewer NeuAc residues. CCS of the $[\text{M}+3\text{H}]^{3+}$ ions displayed a linear trend.

When CCS was plotted vs m/z for the AGP chymotryptic glycopeptide $^{70}\text{TPN}^*\text{KTEDTIF}^{79}$ (NeuAc₀₋₄), the slopes for the tetra-antennary structures were 0.33 for $[\text{M}+4\text{H}]^{4+}$ ions and 0.28 for $[\text{M}+3\text{H}]^{3+}$ ions (Figure S9). The triantennary structure showed a quadratic fit for $[\text{M}+4\text{H}]^{4+}$ ions (NeuAc₀₋₃) and a linear trend for $[\text{M}+3\text{H}]^{3+}$ ions.

For the chymotryptic glycopeptide $^{69}\text{FTPN}^*\text{KTEDTIF}^{79}$, $[\text{M}+4\text{H}]^{4+}$ ions were observed for both tetra- and triantennary structures, whereas $[\text{M}+3\text{H}]^{3+}$ ions were observed only for the triantennary structure (Figure S10). The CCS of these structures were measured as the numbers of NeuAc (Figure S11A) and Gal (Figure S11B) residues were varied stepwise on

the glycopeptide $^{68}\text{YFTPN}^*\text{KTEDTIF}^{79}$. Figure S12 displays CCS vs m/z for the tetra- and triantennary structures of the $[\text{M}+3\text{H}]^{3+}$ to $[\text{M}+5\text{H}]^{5+}$ ions for incremental changes in NeuAc (Figure S12A) and Gal residues (Figure S12B) on the AGP chymotryptic glycopeptide, $^{67}\text{FYFTPN}^*\text{KTEDTIF}^{79}$. As the number of residues of a specific glycan residue was sequentially varied, within a given charge state, the chymotryptic glycopeptides displayed CCS similar to the tryptic glycopeptides discussed above.

Analysis of Deutero-Reduced Permethylated *N*-Glycans and Deglycosylated/Deamidated Peptides

The same type of analysis was carried out for the deutero-reduced permethylated *N*-glycans and the corresponding deglycosylated/deamidated peptides (CCS values, Tables S2 and S3, respectively). Linear trends were observed when CCS values of the derivatized glycans were plotted vs m/z (Figure S13). For the derivatized high mannose *N*-glycans (Figure S13A), the CCS values were 395–538 Å², with a slope of 0.27 in the line for CCS vs m/z . The CCS values of the derivatized complex *N*-glycans and the shortened glycans obtained via glycosidase digestions were also measured. $[\text{M}+3\text{H}]^{3+}$ ions were observed for both tetra- and triantennary structures. The slopes in the plots for CCS vs m/z with variation in NeuAc residues were similar to slopes observed for the glycopeptides (tetra-antennary, 0.34; triantennary, 0.39). For the $[\text{M}+2\text{H}]^{2+}$ ions of the tri- and biantennary structures, the slopes for the lines reporting CCS vs m/z were 0.20 and 0.25, respectively. As the number of terminal Gal residues varied, the CCS values changed at rates similar to those observed for the glycopeptides. For ions corresponding to structures that differed in the number of Gal residues (Figure S13C), the observed slopes were tetra-antennary $[\text{M}+3\text{H}]^{3+}$ ions, 0.40; triantennary 0.39, and for $[\text{M}+2\text{H}]^{2+}$ ions, were tetra-antennary, 0.24; triantennary, 0.27; biantennary, 0.23. The deglycosylated/deamidated peptides did not exhibit significant conformational heterogeneity and yielded well-defined, narrow CCS distributions; a typical example is shown in Supporting Information Figure S14.

Combination of Glycopeptides, Deutero-Reduced Permethylated *N*-Glycans, and Deglycosylated/Deamidated Peptides

To enable visualization of the characteristic separations of the three classes of biomolecules, we assembled these data and plotted the CCS values vs m/z , as shown in Figure 6. For charge states $[\text{M}+2\text{H}]^{2+}$ and $[\text{M}+3\text{H}]^{3+}$, it appears that the average densities differ in the order glycopeptides > peptides > permethylated glycans. The differences in CCS values between the classes of biomolecules are due to class-specific structural folding (dependent on the particular intramolecular folding forces). Although both the deutero-reduced permethylated glycans and the glycopeptides may possess highly branched structures, the deutero-reduced permethylated glycans have larger CCS values than the glycopeptides with similar m/z values, probably because the peptide portions of the latter serve as the major reservoirs of charge localization, and thus the glycan structure does not need to expand its cross section quite so much to accommodate the higher charge states. It is also possible that the attachment of the glycan to the peptide causes compaction of the glycopeptide, and therefore the resulting CCS values for the glycopeptide and corresponding glycan moiety were found to be similar, as shown in the Supporting Information.

Conclusions

With coupled IM-MS, we determined CCS values for glycopeptides resulting from tryptic or chymotryptic digestion of a set of well-characterized glycoprotein standards (bovine RNase B, human transferrin, bovine fetuin and human α_1 -acid glycoprotein), the deuterio-reduced permethylated derivatives of the *N*-linked glycans released from these sources, and the corresponding deglycosylated/deamidated peptides. Multiple charge states and conformations were observed for the glycopeptides and derivatized glycans. We calculated CCS values for the glycopeptides generated by proteolysis, the deglycosylated/deamidated peptides and deuterio-reduced permethylated glycans, including shorter glycoforms that occur naturally or that resulted from stepwise application of exoglycosidases. We determined a linear dependence for all $[M+3H]^{3+}$ ions and for a majority of $[M+4H]^{4+}$ ions, when the CCS values were plotted vs m/z for glycoforms that differed from one another by known increments. The most highly consistent result was the overlapping linear fits observed for the complete $[M+3H]^{3+}$ series for glycoform sets constructed from a given glycopeptide or deuterio-reduced permethylated *N*-glycan. For some sets of glycoforms in $[M+4H]^{4+}$ and $[M+5H]^{5+}$ charge states, quadratic curves were observed. Our results from analysis of the $[M+3H]^{3+}$ ions indicate that the rate of change in CCS upon increments of individual saccharide units is independent of peptide length. The monosaccharide incremental changes in CCS values for both $[M+3H]^{3+}$ and $[M+4H]^{4+}$ glycopeptide ions were 22 to 30 (± 5) Å² per NeuAc residue, 11 to 19 (± 5) Å² for Gal, and 13 to 15 (± 5) Å² for GlcNAc. For both $[M+2H]^+$ and $[M+3H]^+$ deuterio-reduced permethylated glycans, the monosaccharide incremental change in CCS value was 32 to 49 (± 7) Å² per NeuAc residue, 23 to 28 (± 2) Å² for Gal, and 19 to 44 (± 9) Å² for Man. Future work will utilize reference glycans with specific linkages and additional proteases and will examine the behavior of *O*-linked glycopeptides and released *O*-glycans, as well as negative ions, and will refine the results by incorporating tandem MS data and, when possible, molecular dynamics calculations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A two-dimensional plot displaying m/z as a function of drift time represented with a false color scale (least intense features in blue; most intense in red) for the RNase B tryptic glycopeptide $^{34}\text{N}^*\text{LTK}^{37}$. To the left and above the 2-D plot are the mass and drift time spectra, respectively, for $\text{Man}_{5-9} [\text{M}+2\text{H}]^{2+}$ glycopeptide ions.

Figure 2.

(A) Extracted drift time distributions for the $\text{Man}_{5-9} [\text{M}+2\text{H}]^{2+}$ ions observed for the tryptic glycopeptide $^{34}\text{N}^*\text{LTK}^{37}$ from RNase B. (B) CCS values were plotted as a function of m/z for the high mannose glycoforms. Error bars: standard deviations obtained from triplicate measurements.

Figure 3.

Plots displaying CCS vs m/z for the two bi-antennary complex *N*-linked tryptic glycopeptides from transferrin. The left and right columns correspond to variations in the number of each type of terminal saccharide unit on $^{421}\text{C}(\text{Cam})\text{GLVPVLAENYN}^*\text{K}^{433}$ and $^{622}\text{QQQHLFGSN}^*\text{VTDC}(\text{Cam})\text{SGNFC}(\text{Cam})\text{LFR}^{642}$, respectively, after each round of treatment with exoglycosidase. A and D display CCS measurements for products with incremental changes in the number of NeuAc residues; B and E show changes in Gal; and C and F vary the number of GalNAc. Blue diamonds, compact $[\text{M}+4\text{H}]^{4+}$ ions; red squares, elongated $[\text{M}+4\text{H}]^{4+}$ ions; green triangles, $[\text{M}+3\text{H}]^{3+}$ ions. Error bars: standard deviations from triplicate measurements.

Figure 4.

Plots displaying the CCS vs m/z for variations in the number of terminal (A) NeuAc and (B) Gal residues on the triantennary structure of the glycopeptides ¹⁵⁶N*DSRVVHAVEVAL¹⁶⁸ and ¹⁴⁵LC-(Cam)PDC(Cam)PLLAPLN*DSR¹⁵⁹, resulting from proteolytic digestion of fetuin with trypsin (solid symbols) or chymotrypsin (open symbols). Blue diamonds, [M+4H]⁴⁺ ions; green triangles, [M+3H]³⁺ ions. Error bars: standard deviations obtained from triplicate measurements.

Figure 5.

(A) Display of the change in CCS with incremental changes in the number of NeuAc residues present on the [M+4H]⁴⁺ and [M+3H]³⁺ ions of tetra-antennary and triantennary glycoforms of the AGP chymotryptic glycopeptide ⁹⁸NVQREN*GTISRY¹⁰⁹, with CCS plotted vs m/z. Solid and open blue diamonds correspond to [M+4H]⁴⁺ ions of the tetra-antennary and triantennary structures, respectively. For the [M+3H]³⁺ ions, solid and open green triangles correspond to the tetra-antennary and triantennary structures, respectively. (B) A plot displaying CCS vs m/z for [M+3H]³⁺ ions in the series of NeuAc₀₋₃ glycoforms of the triantennary glycopeptides from AGP ¹⁰²EN*GTISR¹⁰⁸ (tryptic peptide, open green triangles) and ⁹⁸NVQREN*GTISRY¹⁰⁹ (chymotryptic peptide, solid green triangles). Error bars: standard deviations resulting from triplicate measurements.

Figure 6.

CCS plotted as a function of m/z for three classes of biomolecules: deuterio-reduced, permethylated glycans, deglycosylated/deamidated peptides, and glycopeptides for (A) $[M+2H]^{2+}$ ions and (B) $[M+3H]^{3+}$ ions. Solid green triangles, tryptic glycopeptides; open green triangles, chymotryptic glycopeptides. Error bars: standard deviations obtained from triplicate measurements.