Fragmentation of Negative ions from *N*-Linked Glycans: Use of Nitrate Adducts to induce Antenna-Specific Fragmentation

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Overview

1. Negative ion electrospray spectra were recorded from *N*-Linked glycans.
2. $[M - H]^-$ and $[M - 2H]^{2-}$ ions were produced from solutions containing NH_4OH.
3. Many of these ions were unstable and decomposed in the ion source.
4. Stable $[M + X]^-$ ions were produced from X = Cl^-, Br^-, I^- and NO_3^-.
5. Sensitivity was highest for $[M + NO_3]^-$ ions and larger glycans were ionized more efficiently than by formation of $[M + H]^+$ or $[M + Na]^+$ ions.
6. CID produced mainly A-type cross-ring ions and C-type glycosidic ions.
7. Ion formation was initiated by proton abstraction from various OH groups.
8. Specific fragmentations produced ions that were diagnostic for:
   - Composition of the two antennae,
   - Presence or absence of a bisecting GlcNAc,
   - Distribution of fucose residues on the antennae.
   - Linkage of the chitobiose core
9. Doubly charged $[M + 2NO_3]^{2-}$ ions produced fragmentation spectra containing mainly the singly charged ions that were present in the spectra of the $[M + NO_3]^-$ ions.
10. Fragmentation of acidic glycans was governed by proton abstraction from the acidic functional groups resulting in fewer cross-ring cleavages.
12. Where the charge was localized, as with glycans derivatized with 2-aminobenzoic acid, the major cleavages were mainly Y-type providing sequence information.

Introduction

Structural identification of underivatized *N*-linked glycans is normally performed with positive ions whose fragmentation spectra are dominated by B- and Y-type (nomenclature proposed by Domon and Costello [1]) glycosidic ions defining sequence but with few cross-ring cleavage ions defining linkage. Spectra also contain many ions, termed internal cleavage ions, that have involved losses from various sites and which make interpretation difficult. This poster explores the utility of negative ions generated by anion addition and whose fragmentation spectra were found to contain ions defining specific details of the glycan structure.

Materials and Methods

Materials

N-linked glycans were released with hydrazine from the well-characterised glycoproteins ribonuclease B, porcine thyroglobulin, chicken ovalbumin, bovine fetuin, and human α1-acid glycoprotein. Bi- and tri-antennary complex *N*-linked glycans were obtained from the sialylated glycans by desialylation with 10% acetic acid for 30 mins at 80ºC.

Preparation of 2-AB and related derivatives

Derivatives with 2-aminobenzamide (2-AB) were prepared by reductive amination by a modification of the method described by Bigge *et al*. [2].

Electrospray (ESI) mass spectrometry

ESI mass spectrometry was performed with a Waters-Micromass quadrupole-time-of-flight (Q-Tof) Ultima Global instrument (Waters/Micromass Ltd, Manchester). Samples (about 50 pmoles/mL) in 1:1 (v:v) methanol:water containing 0.1 M NH_4OH, or a salt as discussed below, were infused at 5 mL/min. Operating conditions were: ion source temperature, 120ºC; nebulizer gas (N_2) 100ºC, 50 L/hr; cone gas 450 L/hr; infusion capillary, 3.0 kV, argon pressure for CID, 0.5 Bar; collision cell voltage, 80-120 V for the singly charged ions and 30 - 50 V for the doubly charged ions.

Sensitivity measurements

Ribonuclease glycans and their 2-AB derivatives (1 mg, 500 nM of $(GlcNAc)_2(Man)_9$) were diluted successively by 1:5 increments with 1:1 (v:v) methanol:water containing 0.1 mM NH_4OH or NH_4NO_3. Solutions were infused for one min. at each of several concentrations and the total ion current was measured for $(GlcNAc)_2(Man)_5$. Five spectra were acquired at each concentration.

Effect of the RF-1 and collision cell voltages

Glycans were infused at 5 mL/min at about 50 pmoles/mL. Spectra were recorded for 20 secs with an acquisition time of 2 sec (10 scans). For measurements of the effect of the RF-1 potential, this was raised in 10 V steps from 0 to 250 volts and spectra were recorded at each voltage. For measurements of the effect of collision energy, the collision cell voltage was raised in 2 V steps from the voltage at which the fragments started to appear until most ions had fragmented.

Results

Figure 1a shows the negative ion mass spectrum of *N*-linked glycans from chicken ovalbumin ionized $[M - H]^+$ ions from ammonium hydroxide solution. Considerable in-source fragmentation occurred. Figure 1b shows the stabilizing effect ammonium nitrate and the formation of nitrate adducts. Stronger signals were produced from the larger glycans than were observed by other ionization methods such as formation of $[M + Na]^+$ ions (Figure 1c). No significant in-source fragmentation was observed from the nitrate adducts unlike $[M - H]^-$ ions produced from solutions without nitrate. The effect of other salts, such as ammonium chloride and sodium iodide were also investigated but the best results were obtained from ammonium nitrate. Detection limits were of the order of 50 fmoles.

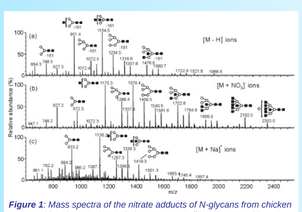


***Figure 1**: Mass spectra of the nitrate adducts of N-glycans from chicken egg white glycans. Open circle = mannose, filled square = GlcNAc*

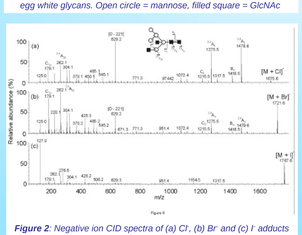


***Figure 2**: Negative ion CID spectra of (a) Cl⁻, (b) Br⁻ and (c) I⁻ adducts of a hybrid N-linked glycan.*

The $[M + Adduct]^-$ ions fragmented in an identical manner to the $[M - H]^-$ ions suggesting that, in all cases, the initially-formed ion was of the type $[M - X]^-$ and that this ion eliminated HX. The proton could be extracted from any OH group to leave an ion with a localised charge which then fragmented in a specific manner to yield structurally-diagnostic fragment ions. This behaviour was different from that observed in positive ion mode where many fragment ions were formed by several pathways. Adducts with Cl^-, Br^-, and NO_3^- fragmented well, those with I^- showed little fragmentation Figure 2), adducts with F^- fragmented extensively in the ion-source.

Figure 3 shows the fragmentation spectrum of the high-mannose glycan $(Man)_9(GlcNAc)_2$. Fragmentation of the chitobiose core gave ions at [M - XH - 161]⁻ (*m/z* 1072.5) and [M - XH - 364]⁻ (*m/z* 869.4) attributed to $^{2,4}A$ cleavages. Formation of these ions can be rationalised by the mechanism shown in Scheme 1. These ions did not shift when fucose was attached to position 6 of the reducing-terminal GlcNAc and they also defined the 1-4 linkage of the two GlcNAc rings. Mechanisms of this type account for most of the abundant cross-ring cleavage ions observed in negative ion spectra.

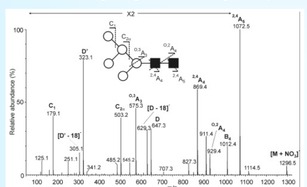
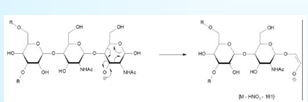
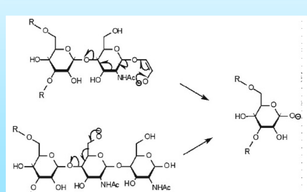


***Figure 3**: Negative ion CID spectrum of the high-mannose glycan $(Man)_9(GlcNAc)_2$.*



***Scheme 1**: Proposed mechanism for the formation of $^{2,4}A$ ions.*



***Scheme 2**: Proposed mechanisms for the production of C-type ions (C_3 from $(Man)_9(GlcNAc)_2$)*

C-type ions (Scheme 2) were present from cleavages at the reducing terminus and allowed the sequence of the antennae to be traced. However, those produced by cleavages nearer the reducing terminus were only visible at reduced collision energy. Several other ions, such as the $^{0,2}A_2$ fragment from the reducing terminus were also visible only at low collision energy (Figures 4 and 5) as they readily fragmented further as the energy was raised. Thus, spectra recorded at low energy are important for revealing fragments that are intermediates in the formation of the main diagnostic ions. Fragmentation patterns changed further at higher collision energy to give more abundant ions revealing sequence (Figure 4c).

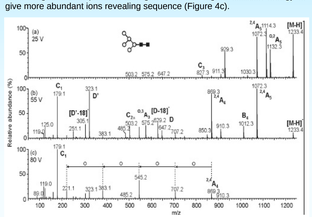


***Figure 4**: Negative ion CID spectra of the high-mannose glycan $(Man)_9(GlcNAc)_2$ recorded at three collision energies.*

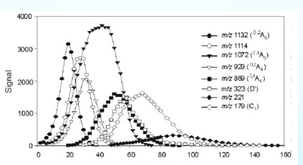
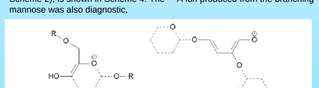


***Figure 5**: Variation of signal strength with collision energy for fragments in the negative ion spectrum of $(Man)_9(GlcNAc)_2$.*

Several of the ions present in these negative ion spectra were diagnostic of specific structural details of the *N*-linked glycans as the result of their formation from abstraction of specific protons.

Ions diagnostic for antenna composition:

The ion labelled as D in these spectra was specific to the composition of the 6-antenna and a proposed mechanism for its formation from the C-ion (see Scheme 2), is shown in Scheme 4. The $^{0,3}A$ ion produced from the branching mannose was also diagnostic,



***Scheme 3**: Proposed structures of the $^{0,3}A$ and E ions.*

Complex glycans (Figure 5) gave prominent D ions defining the 6-antenna and E ions (Scheme 3) that defining the 3-antennae. A prominent ion at *m/z* 424.2 containing the galactose and GlcNAc residues from the antennae plus 59 mass units. Formation of this ion can be rationalized by mechanisms similar to that shown in Scheme 1 where the abstracted proton originates from an OH group adjacent to the linkage position.

The substitution pattern of the 6-antenna in the high-mannose glycans was the same as that of the core mannose (3,6-links). Therefore, a similar fragmentation pattern might be expected. This was seen in the production of the D' and $[D'-18]^-$ ions (*m/z* 323.1 and 305.1 respectively, Figure 3).

Ion diagnostic for a bisecting GlcNAc

Spectra of glycans containing a bisecting GlcNAc residue (4-linked to the core mannose) produced a very abundant ion at $[D - 221]^-$ in place of the $[M - 18]^-$ ion (Figure 6). The 221 mass loss was that of the bisecting GlcNAc.

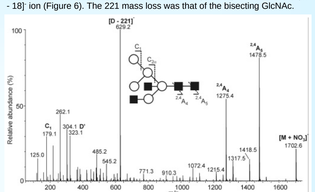
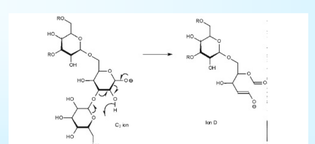


***Figure 6**: Negative ion CID spectrum of the bisected hybrid glycan from chicken ovalbumin.*



***Scheme 4**: Proposed mechanism for the formation of Ion D, diagnostic for the composition of the 6-antenna*

The 1-4 linkage of the Gal-GlcNAc bond produced the $^{0,2}A_2$ and $[^{0,2}A_2 - 18]^-$ ion pair whose formation could again be rationalized by the mechanism shown in Scheme 1. Confirmation of the loss of a proton from the 3-position of the GlcNAc residue was provided by the absence of these ions from the spectra of biantennary glycans containing two fucose residues attached to the 3-positions of the GlcNAc residues (see Figure 9).

Substituents on the antennae of the complex glycans produced the appropriate shifts in the masses of the diagnostic D and E ions (Figure 7). Thus, because a single α-galactose substituent on the antennae of a biantennary glycan from porcine thyroglobulin (Figure 8a) produced only partial shifts of ions D and E, it can be concluded that the spectrum is a mixture of isomers in which the α-galactose can be attached to either antennae, in contrast to published work [3] that suggests that it is only on the 6-antenna. The mass of the $^{2,4}A_5$ ion shows that the fucose is substituted at the 6-position of the reducing terminal GlcNAc residue. In the spectrum of the monofucosylated triantennary glycan from human α1-acid glycoprotein shown in Figure 8b, the fucose is substituted on the 3-antenna. Thus, the $^{2,4}A_5$ ion only shows a loss of 161 mass units (from the mass of the $[M - HNO_3]^-$ ion whereas that of the E ion, diagnostic for the composition of the 3-antenna shifts from *m/z* 831.3 (see Figure 7b) to *m/z* 977.3. The D ion remains at *m/z* 688.3. The ion at *m/z* 242.1 (Gal-GlcNAc plus 59) partially shifts to *m/z* 586.1 and 570.1 in the two spectra, respectively reflecting the addition of a galactose residue (Figure 8a) or fucose substituent (Figure 8b) respectively on one of the antennae.

The position of multiple fucose substituents, as in the biantennary glycans from human parotid glands (Figure 9) could also be established.

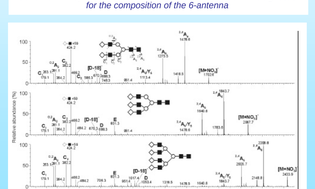


***Figure 7**: Negative ion CID spectra of the nitrate adducts of complex N-linked glycans. Open diamond = galactose.*

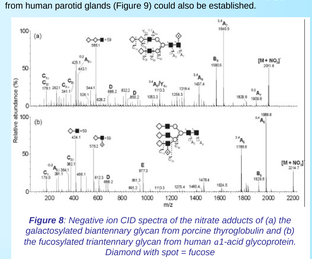


***Figure 8**: Negative ion CID spectra of the nitrate adducts of (a) the galactosylated biantennary glycan from porcine thyroglobulin and (b) the fucosylated triantennary glycan from human α1-acid glycoprotein. Diamond with spot = fucose*

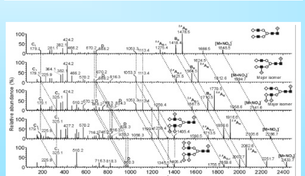


***Figure 9**: Negative ion CID spectra of fucosylated biantennary glycans from human parotid glands. The broken lines show movement of some of the ions defining the positions of the fucose residues.*

Conclusions

Negative ions from *N*-linked glycans were produced efficiently from solutions containing several anions. Some of these anions, such as Cl^-, Br^-, I^- and NO_3^- gave stable adducts of the type $[M + X]^-$ and $[M + 2X]^{2-}$. The larger glycans that had earlier proved to be problematical in positive ion mode could be ionized well by this method. The best results in terms of stability and signal strength were produced by addition of ammonium nitrate to the electrospray solvent to form $[M + NO_3]^-$ ions. Anions such as F^- produced adducts that were relatively unstable and fragmented in the ion source. Addition of NH_4OH gave what are presumed to be hydroxyl adducts that, again, fragmented within the ion source.

The $[M + X]^-$ ions fragmented under CID conditions by loss of HX to give a $[M - H]^-$ ion with proton abstraction from several OH groups of the carbohydrate. These ions fragmented by cross-ring cleavages and formation of C-type glycosidic fragments in direct contrast to positive ions that mainly produced B and Y fragments. This behaviour can be rationalized by the localized negative charge in the $[M - H]^-$ ion feeding electrons into the sugar rings, causing then to cleave. Electron transfers could be very extensive; spectra acquired at low collision cell energy often produced ions whose formation could be rationalized by proposing that the electron movements ceased earlier than they did at higher energy, accounting for the fact that some ions present at low energy disappeared as the energy was raised.

Doubly charged ions fragmented in a very similar manner to their singly charged counterparts with the production of mainly singly charged fragments. Thus, fragmentation spectra of the larger glycans could conveniently be obtained by fragmentation of these doubly charged ions.

The abstraction of specific protons by the various anions produced very explicit fragmentation and the production of abundant ions that revealed details of the glycan structure that were difficult to obtain by traditional methods. Examples of these fragment ions were ones that revealed the composition of specific antennae, the presence of bisecting GlcNAc residues, the distribution of substituents such as fucose on the antennae and the structure of several of the linkages.

Sialylated glycans ionized mainly by abstraction of the acidic protons giving ions in higher charge states when several sialic acid groups were present. Fragmentation of these ions was dominated by sialic acid losses resulting in a suppression of the diagnostic ions. However, these could be recovered by methyl ester formation of the carboxy-groups of the sialic acid.

References

1. Domon, B.; Costello, C. E. A systematic nomenclature for carbohydrate fragmentations in FAB-MS/MS spectra of glycoconjugates. *Glycoconjugate* J. 1988, **5**, 397-409.
2. Bigge, J. C.; Patel, T. P.; Bruce, J. A.; Goulding, P. N.; Charles, S. M.; Parekh, R. B. Nonselective and efficient fluorescent labeling of glycans using 2-aminobenzamide and anthranilic acid. *Anal. Biochem*. 1995, **230**, 229-238.
3. de Waard, P.; Kooreveaar, A.; Kamerling, J. P.; Vliegenthart, J. F. G. Structure determination by ^1H NMR spectroscopy of (sulfated) sialylated *N*-linked carbohydrate chains released from porcine thyroglobulin by peptide-*N*^{4}-(*N*-acetyl-β-glucosaminyl)asparagine amidase-F. *J. Biol. Chem*. 1991, **266**, 4237-4243.

Acknowledgements

I thank Professor R. A. Dwek, Director of the Oxford Glycobiology Institute for help and encouragement and the Wellcome Trust for a grant to purchase the Q-Tof mass spectrometer.

A copy of this poster is on the Glycobiology web site at:

http://www.bioch.ox.ac.uk/glycob/index.html