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Note

Resistance to deglycosylation by ammonia of IgA1 O-glycopeptides: implications for the β-elimination of *O*-glycans linked to serine and threonine

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Abstract—Pools of O-glycopeptides (and their deglycosylated analogues) derived from trypsin-digested normal human serum IgA1 have been treated with ammonia under conditions reported to result in complete liberation of *O*-glycans linked to serine and threonine residues in glycopeptides and glycoproteins. MALDI-TOF MS analysis has revealed that only one of the six glycosylated sites is susceptible to β-elimination under these conditions. It is likely that resistance to β-elimination is due to very close proximity of proline to the glycosylated serine or threonine residues. Preliminary results using 0.1 M NaOH (instead of ammonia) to perform β-elimination indicated that there was also selective de-O-glycosylation with this reagent, however, these results were complicated by the concomitant hydrolysis of the peptide bonds. These findings may have implications for similarly O-glycosylated peptides and proteins and possibly for other chemical methods that are used to carry out β-eliminations of *O*-glycans.

Keywords: β-Elimination; Ammonia; O-Glycopeptide; MALDI-TOF MS; IgA

Several chemical methods have been reported for liberating, via β-elimination, glycans that are O-linked at serine or threonine residues within a polypeptide. The classical alkaline β-elimination method[1] uses NaOH (together with $NaBH_4$ to reduce and thereby stabilise the released saccharide) and more recent methods have described the use of hydrazine[2] or ethylamine[3] or ammonia.[4] Ammonia is a useful reagent since it provides products that are readily amenable to MS analysis and can provide information about the positions of O-glycosylation within a polypeptide. β-Elimination using ammonia has recently been applied in (glyco-) proteomic studies by carrying out chemical de-O-glycosylation on gel separated glycoproteins prior to the *in gel* digestion with trypsin.[5] Previous investigations in our laboratory have studied the glycoform composition of O-glycopeptides derived from human serum IgA1 using MALDI-TOF MS.[6,7] As an extension of this work, and in an attempt to obtain information about positions of substitution within the peptide, the de-O-glycosylation of IgA glycopeptides with ammonia was investigated. The results of this study, in particular the selectivity of β-elimination, are presented here. (Recently, electron capture MS has been used to analyse intact O-glycopeptides derived from IgA and this has enabled *O*-glycan substitution to be determined directly without recourse to liberate the glycans by chemical means.[8]) The O-glycopeptides derived from IgA1 used in the present study are shown in Figure 1, the peptide being substituted at 3, 4, 5 or 6 serine and/or threonine residues by mono-, di- or tri- saccharides.[7,9] The 33mer peptide results from an unusual tryptic cleavage on the carboxyl side of a serine adjacent to S-pyridylethylated cysteines (the sequence is –SCC–) and its structure has been confirmed by Edman sequencing[10] and accords

Abbreviations: GalNAc, *N*-acetylgalactosamine; Gal, galactose; NeuAc, *N*-acetylneuraminic acid; MALDI-TOF MS, matrix assisted laser desorption-time of flight mass spectrometry; THAP, 2,4,6-trihydroxyacetophenone

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Figure 1. Primary sequence of the 33mer tryptic peptide derived from human serum IgA1. The numbers refer to residues in the intact IgA1 molecule. Locations are indicated for five glycosylation sites[9] (▲) together with the possible location of the sixth glycosylation site[7] (▼). Cysteine is present as its *S*-pyridylethyl derivative.

with MALDI-TOF MS[6,10] and electrospray MS/MS data (unpublished data). Ammonia actually causes two different modifications to the glycopeptides. One, a novel selective cleavage of the peptide chain between the adjacent asparagine (^{211}N) and proline (^{212}P) residues (Fig. 1), probably proceeding via a succinimide intermediate. (The general nature of N–P cleavage by ammonia has been demonstrated using model peptides.[11]) In addition to this peptide cleavage, the IgA1 glycopeptides also underwent de-O-glycosylation but apparently at only one of the sites substituted with *O*-glycans. Thus most species observed in the MALDI-TOF spectrum of the pool of O-glycopeptides (shown in Fig. 2A) are observed at m/z >5000, whereas after treatment with 25% aqueous ammonia at 45 °C for >72 h new species are mostly observed (Fig. 2B) at m/z <5000 (a loss of $\sim 10^3$ Da). These shifts to lower m/z can be rationalised from both the cleavage of the N–P bond, resulting in the loss of the tetrapeptide HYTN (−515 Da), together with the removal of a single tri-, or di-, or mono-saccharide, that is, NeuAc-Gal-GalNAc (−656 Da), or Gal-Gal-NAc (−365 Da), or GalNAc (−203 Da) giving a total shift of up to 1171 Da. Several examples in keeping with this proposal are apparent from a comparison of Figure 2A with B. As depicted in Figure 2A, there are major ions at m/z 6014, the peptide being substituted at 5-4-3 (this code is used throughout this report to indicate, respectively, the number of GalNAc-Gal-NeuAc residues present in the glycopeptides) m/z 5723 (5-4-2), m/z 5431 (5-4-1), m/z 5228 (4-4-1) and ions corresponding to a loss of ~1171 Da in Figure 2B at m/z 4841, 4552, 4262, 4059. The greater complexity of the spectrum of the ammonia-treated pool is to be expected since, from a single ion present in Figure 2A, several new species may be produced because of possible micro-heterogeneity at a glycosylated site. In addition some types of substitutions may not be susceptible to β-elimination, such species only undergoing peptide cleavage. This could explain the relatively intense ions observed at m/z 4100 and 3897 (* Fig. 2B) which could arise from stable 4-2-0 and 3-2-0 species, undergoing loss of tetrapeptide (−515 Da) only. (These ions could also arise from 5-2-0 and 4-2-0 substitution (−515 (HYTN) and −203 (GalNAc)). It is also reported that following β-elimination, addition of ammonia to the so-formed double bond can be impeded/prevented because of steric hindrance from adjacent prolines; threonine residues may also undergo loss of CH_2=CHOH.[4] Despite the complexity of the spectrum in Figure 2B, it is apparent that most of the oligosaccharide chains in the IgA1 glycopeptide pool are resistant to β-elimination under the conditions used and this is the case even after prolonged treatment (>7 days at 45 °C) with 25% ammonia after which time a similar spectrum to that depicted in Figure 2B is observed. A clearer picture emerges when the desialylated glycopeptide pool is similarly examined. These spectra are shown in Figure 2C and D and in this instance, species are shifted in the latter spectrum by −880 Da which can be rationalised by loss of the tetrapeptide together with the single disaccharide Gal-GalNAc (−515 −365 Da). For example, there are ions in Figure 2C at m/z 5141 (5-4-0) and 4938 (4-4-0) that correspond to those appearing at 4262 and 4059 in Figure 2D. There are also ions whose m/z accord with penta-substitution such as that at m/z 4465 (5-3-0), 4302 (5-2-0) (* in Fig. 2D). These could have arisen from species resistant to ammonia (−515 HYTN only) or could also arise from additional β-elimination from a single site of hexa-substituted species. As is the case with the native pool described above, even after similar prolonged treatment of the desialylated sample (and also of those mentioned below) no further significant change was evident. Comparison of the desialylated and degalactosylated pool of oligosaccharides (the peptide now being only substituted by GalNAc) without or with ammonia treatment (Fig. 2E and F) indicates that after treatment some ions are shifted by −718 Da (−515 −203); m/z 4494 and 4291 to 3778 and 3575. Penta-substituted species (e.g., m/z 3980, * in Fig. 2F) appear to be stable, again suggesting that in some species the five occupied sites are resistant to ammonia. Inspection of spectra in the early stages demonstrated that deglycosylation and cleavage of the N–P bond appeared to occur at a similar rate. Finally for the totally deglycosylated species (m/z 3476, Fig. 2G) treatment with ammonia causes the peptide to be converted, as a result of N–P cleavage, into new species m/z 2961 (Fig. 2H) and m/z 534 (data not shown). The results reported here clearly demonstrate that 5 of the 6 sites that are substituted with *O*-glycans in IgA1 glycopeptide are resistant to β-elimination with ammonia. The reactivity of *O*-glycans towards β-elimination with ammonia would be expected to be affected by peptide structure. Serine/threonine residues at positions 228, 230, 236 and 238 (Fig. 1) are the most sterically hindered because they have two adjacent prolines and consequently, if O-glycosylated, would probably be expected to be least susceptible to β-elimination.

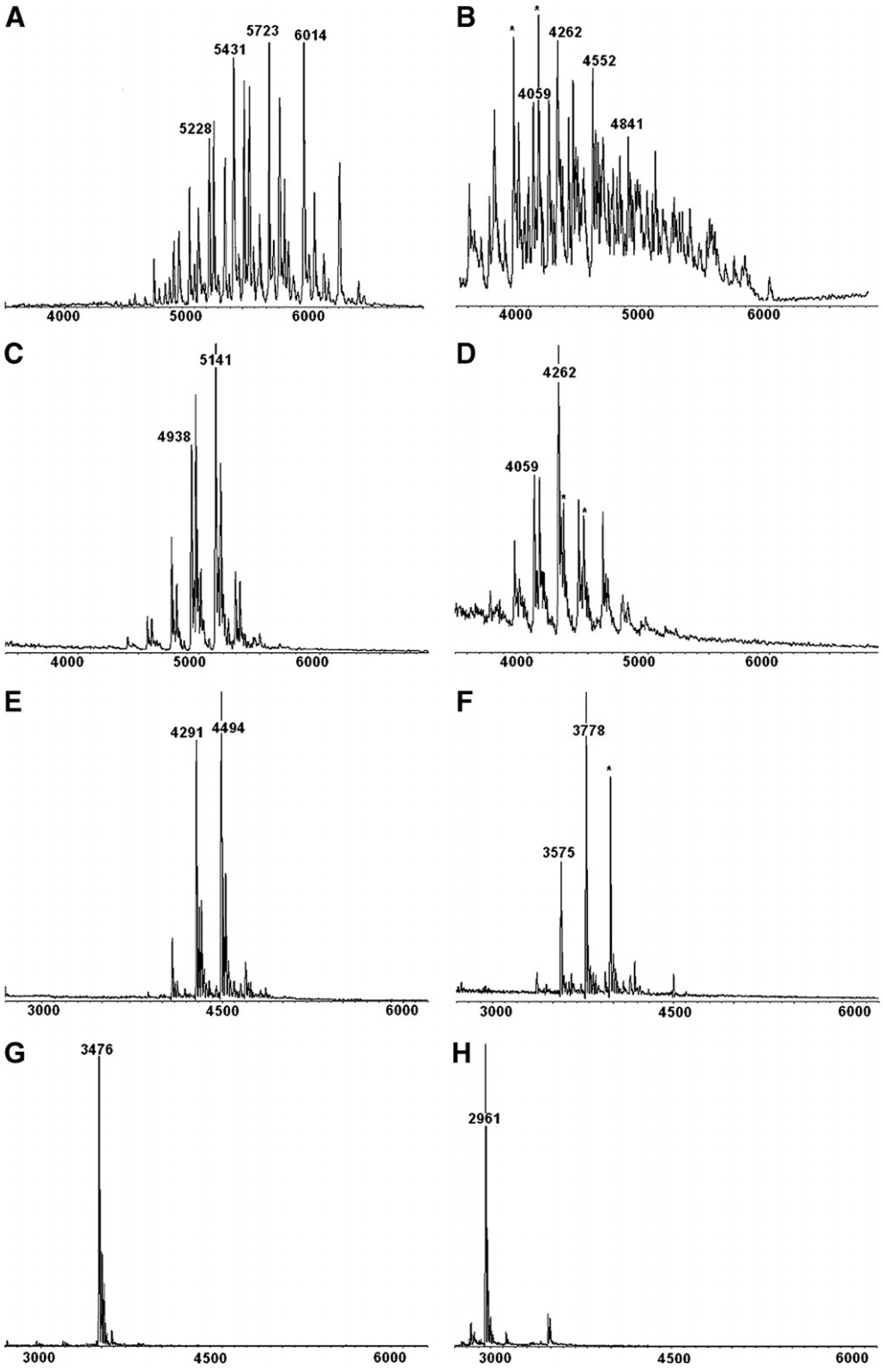

Figure 2. MALDI-TOF mass spectrum, in linear mode, of: A—normal human serum IgA1 O-glycopeptides; B—sample from A after treatment with 25% ammonia at 45 °C for 72 h; C—sample from A after treatment with neuraminidase; D—sample from C after treatment with 25% ammonia at 45 °C for 72 h; E—sample from C after treatment with β-1,3 galactosidase; F—sample from E after treatment with 25% ammonia at 45 °C for 72 h; G—sample from E after treatment with α-*N*-acetylgalactosaminidase; H—sample from G following after treatment with 25% ammonia at 45 °C for 72 h *m*/*z* values are shown on *x*-axes and $[M+H]^+$ average masses are used throughout. 2,4,6-Trihydroxyacetophenone (THAP) 3 mg/mL in 1:3 CH_3CN: 50 mM aqueous dibasic ammonium citrate was used as a matrix. Salt adducts, $[M+Na]^+$, etc. are present in the spectra of B–G. *Correspond to penta-substituted species—see text for details.

Of the remaining sites, 232 may be the next most resistant, as it has one immediate and a number of very close proline neighbours. At this time, however, in the absence of firm data, the precise details of which these are can only be a conjecture. Preliminary results using 0.1 M NaOH (in place of ammonia) at 45 °C to carry out β-elimination of the desialylated and degalactosylated IgA glycopeptides (cf. Fig. 2E) resulted in a relatively rapid (within 30 min) loss of one molecule of GalNAc (−220 Da), as shown by MALDI-TOF MS, with major ions being observed *inter alia* at *m*/*z* 4070 and 4273 corresponding, respectively, to 4-0-0 and 3-0-0 *dehydro* species. After 1 h, additional ions were present at *m*/*z* 3555 and 3758 (corresponding to the further loss of the tetrapeptide HYTN, −515 Da) and these latter species in which only a single GalNAc had been removed were still present as major ions even after 8 h incubation. The reaction mixture was also becoming more complex as a result of further hydrolysis of peptide bonds; however, two other ions were prominent in the spectrum at *m*/*z* 2721, 2518 and these corresponded with glycopeptides (*dehydro* 4-0-0 and 3-0-0) resulting from peptide chain fission on the carboxyl side of the pyridylethylated cysteine residue, that is, from glycosylated ^{221}PVPSTPP-TPSPSTPPTPSPS^{240} (Fig. 1). Because of the increasing complexity of the spectra, this reaction was not further investigated by MALDI-TOF MS. From these preliminary results it would appear that with NaOH, the IgA glycopeptides also exhibit selective β-elimination not dissimilar to that described for ammonia, except that in the case of NaOH, elimination of a single glycan precedes fission of the N–P bond. Further experiments will be required to establish the precise nature of these reactions. In conclusion, the use of ammonia (and possibly NaOH) to perform β-elimination of glycans O-linked to serine or threonine will require the selectivity described here to be considered when such reactions are carried out on other glycopeptides and glycoproteins. This may also be the case when other reagents are used to carry out β-eliminations. Further studies with IgA1 and other model systems should provide further insight into the structure-selectivity relationship for the chemical deglycosylation of glycans when they are O-linked to serine and threonine residues within polypeptides.

1. Experimental

IgA glycopeptides and their deglycosylated analogues were prepared, and MALDI-TOF MS analyses were carried out as described previously.[6]

Elimination reactions using NH_4OH or NaOH were performed as described.[4] For the reaction products from NaOH incubations, and prior to MALDI-TOF MS, the solution was neutralised with HOAc and then desalted using a ziptip® (Millipore, Bedford, USA) following the manufacturer's instructions.

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