Glycoconjugate Journal (1994) **11**:180–186

Characterization of a single glycosylated asparagine site on a glycopeptide using solid-phase Edman degradation

ANDREW A. GOOLEY[1], ANTHONY PISANO[1], NICOLLE H. PACKER[1], MALCOLM BALL[1], ALUN JONES[2], PAUL F. ALEWOOD[2], JOHN W. REDMOND[1] and KEITH L. WILLIAMS[1]*

[1] *Macquarie University Centre for Analytical Biotechnology (MUCAB), Macquarie University, Sydney, NSW, 2109, Australia*

[2] *The Centre for Drug Design and Development, University of Queensland, St. Lucia, Brisbane, QLD, 4072, Australia*

Received 3 May 1994, revised 13 June 1994

The characterization of site-specific glycosylation is traditionally dependent on the availability of suitable proteolytic cleavage sites between each glycosylated residue, so that peptides containing individual glycosylation sites are recovered. In the case of heavily glycosylated domains such as the *O*-glycosylated mucins, which have no available protease sites, this approach is not possible. Here we introduce a new method to gain site-specific compositional data on the oligosaccharides attached to a single amino acid. Using a model glycopeptide from a mutant human albumin Casebrook, glycosylated PTH-Asn was recovered after sequential solid-phase Edman degradation, subjected to acid hydrolysis and the sugars were identified by high performance anion exchange chromatography with pulsed amperometric detection. The PTH-Asn(Sac) derivative was further characterized by ionspray mass spectrometry. Comparison between an endoproteinase Glu-C glycopeptide and a tryptic glycopeptide showed that the oligosaccharide attached to Asn494 was stable after at least 10 cycles of Edman degradation.

Keywords: N-terminal sequencing; glycoprotein; glycan analysis; covalent immobilization; mass spectrometry

Introduction

The enzyme glycosylation of glycoproteins can be divided into three principal classes [1]: (i) *N*-glycosylation, the linkage between carbohydrate and the amide of asparagine; (ii) **O**-glycosylation, the linkage between carbohydrate and the hydroxyl oxygen of serine or threonine (and to a lesser extent hydroxy-proline and hydroxy-lysine); and (iii) the glycosylphosphatidylinositol (or GPI) anchor, a class of glycolipid linked to the C-terminal amino acid of protein via ethanolamine phosphate.

To determine the structure of carbohydrates attached to a polypeptide, the traditional approach has been to release and separate the oligosaccharides from the protein backbone and then determine which sugars are present, their linkages and finally the sequence of the individual oligosaccharides [1]. The advent of high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) in the 1980s, provided a more satisfactory method for determining the extent of oligosaccharide heterogeneity and constitution compared with older techniques [2–4]. An alternative approach, fluorophore assisted carbohydrate electrophoresis (FACE™), provides improved sensitivity for oligosaccharide and monosaccharide constitutional analysis, with the additional advantage of the high resolution of polyacrylamide gel electrophoresis [5].

The problem with the above approaches is that many glycoproteins contain a heterogeneous collection of both *N*-linked and *O*-linked oligosaccharides, which often, for example in the 'mucin-like' *O*-glycosylated proteins, occurs at many sites in a clustered domain. Hence chemical or enzymic treatment of a glycoprotein/glycopeptide releases a pool of carbohydrate structures, and may provide no specific information concerning glycosylation at any particular site. Mass spectrometry (MS), and in particular electrospray ionization MS (ESI-MS), has been increasingly used in the identification and characterization of glycopeptides [6]. The technique involves the separation of glycopeptides by liquid chromatography and subsequent identification of glycopeptides by tracking the diagnostic sugar oxonium-ions induced by collision fragmentation [6]. However, the limitations of MS analysis are that the technique cannot distinguish what type of sugars are at any one particular site (such as the different hexoses and

* To whom correspondence should be addressed.

hexosamines) or which sites are glycosylated in a clustered domain. As a result, ESI-MS has been largely restricted to *N*-linked glycoproteins/peptides, and it has not been possible to study *O*-glycosylated domains, such as those found in mucins [7], the immunoglobulin superfamily molecules CD8 and IgA [8, 9] and κ-casein [10].

Recently we have shown that glycosylated PTH-amino acids can be recovered during the normal Edman degradation procedure using a solid-phase protein sequenator [11, 12]. However, it was not established how much sugar remained attached to a particular amino acid during the acid cleavage of the N-terminal amino acid from the phenylthiocarbamyl(PTC)-peptide and acid conversion of the anilinothiazolinone(ATZ)-amino acid to the phenylthiohydantoin(PTH)-amino acid.

Solid-phase Edman degradation provides the ideal chemical method for isolation of individual glycoforms as it proceeds normally through *N*-glycosylated and *O*-glycosylated amino acids [11], including extensively *O*-glycosylated domains such as the N-terminus of glycophorin A [12]. Since Edman degradation results in the sequential removal of amino acids from the N-terminus, in principle it is possible to collect each glycosylated amino acid in a complex glycopeptide and subject it to carbohydrate analysis. Here we show for the first time, by using a model glycopeptide from the human albumin mutant Casebrook, that it is possible to collect the PTH-derivative of glycosylated asparagine (PTH-Asn(Sac)), from the sequenator (with high recovery of the sugar derivative) and obtain neutral sugar compositional data using HPAEC-PAD and mass data using ESI-MS.

Materials and methods

Materials

Human Casebrook serum albumin was kindly donated by Dr S. Brennan, University of Christchurch, New Zealand. Concanavalin-A Sepharose was purchased from Pharmacia. PTH-amino acid standards were from Applied Biosystems Inc. (Ca, USA). Trypsin (type XIII TPCK treated) was from Sigma. Endoproteinase Glu-C was from Boehringer-Mannheim Ltd. All other chemicals and reagents were of analytical grade.

Preparation of Casebrook albumin tryptic glycopeptide Arg485-Lys500

The Casebrook albumin tryptic glycopeptide was purified by Concanavalin-A Sepharose affinity chromatography using a method previously described by Peach and Brennan [13]. Briefly, 10 mg of Casebrook albumin was reduced and alkylated with N-iodoacetyl-N-(5-sulfo-1-naphthyl)ethylenediamine (AEDANS) according to Gorman [14]. The reduced and alkylated Casebrook albumin was dialysed against 100 mM Tris/HCl pH 8.0 and then digested with trypsin (4% w/w) for 16 h at 37 °C. The Casebrook albumin tryptic digest was loaded on to a Concanavalin-A Sepharose affinity column and washed with 10 ml of 25 mM NH_4HCO_3: 0.5 mM $MgCl_2$:0.5 mM $CaCl_2$:0.5 mM $MnCl_2$ and the tryptic peptide was eluted with 1.5 ml fractions of 25 mM NH_4HCO_3:0.5 mM $MgCl_2$:0.5 mM $CaCl_2$:0.5 mM $MnCl_2$ and 250 mM α-methyl glycoside. The first 3 ml were found to contain fluorescence, due to the AEDANS labelling of Cys487 of the tryptic glycopeptide. The tryptic glycopeptide was purified on reversed phase HPLC (SMART™ Pharmacia Biotech) using a C_{18} Sephasil™ column (2.1 mm × 100 mm, Pharmacia Biotech); solvent A: 0.1% (v/v) TFA + water; solvent B: 90% (v/v) acetonitrile + 0.1% (v/v) TFA using a two-step linear gradient of: 0–30% B over 10 min then 30–80% B over 10 min with a flow rate of 100 μl min^{-1}. The chromatography was monitored at 214 nm. The tryptic glycopeptide was the only major peak and it eluted at approximately 11 min (data not shown).

Desialylation of Casebrook albumin tryptic glycopeptide Arg485-Lys500

Approximately 10 nmol of Casebrook albumin tryptic glycopeptide was freeze-dried then re-dissolved in 200 μl of 0.1 M trifluoroacetic acid (TFA) and incubated at 80 °C for 40 min to remove sialic acid. The desialylated glycopeptide Arg485-Lys500 was freeze dried and re-dissolved in 200 μl 10% (v/v) acetonitrile then further purified by reversed phase chromatography as described above.

Preparation of Casebrook albumin V8 treated tryptic glycopeptide: Val493-Asn(Sac)494-Glu495

Six nmol of Casebrook albumin tryptic glycopeptide, Arg485-Lys500, was vacuum concentrated close to dryness on a Savant Speed-Vac, then dissolved in 100 μl of 50 mM ammonium bicarbonate (pH 8.9). The digestion of Casebrook glycopeptide Arg484-Lys500 was commenced with the addition of 10 μg endoproteinase Glu-C (V8). The sample was incubated for 17 h at 37 °C. The Casebrook V8 glycopeptides were purified using the conditions described above for the tryptic peptide. The chromatogram consisted of four peaks identified by sequence analysis as: (1) Val493-Asn(Sac)494-Glu495, retention time (t_r) 8 min; (2) Thr496-Tyr-Val-Pro-Lys500, t_r 12 min; (3) Arg485-Pro-Cys-Phe-Ser-Ala-Leu-Glu492, t_r 17.5 min; (4) V8 endoproteinase, t_r 20 min (data not shown).

Covalent attachment of Casebrook glycopeptide Arg485-Lys500 and Val493-Asn(Sac)494-Glu495

The desialylated Casebrook tryptic or V8 glycopeptides (for amounts see Figure legends, Figs 1–3) were covalently attached to Sequelon-AA™, an arylamine activated polyvinylidene difluoride (PVDF) membrane disc, using water soluble carbodiimide. The covalent attachment of the desialylated Casebrook glycopeptides was carried out at 4 °C to increase coupling yield [15] following the covalent

attachment method provided in the Reagent Kit User's Guide (manufacturer's instructions, MilliGen/BioSearch, Division of Millipore, Milford, USA).

Solid-phase Edman degradation

The covalently bound Casebrook glycopeptides coupled to Sequelon-AA™ discs were subjected to automated solid-phase Edman degradation on the MilliGen ProSequencer™ 6600 using the standard program supplied by the manufacturer. The PTH-derivatives of amino acids and glycoamino acids were transferred directly from the conversion flask to a Waters 600 LC system equipped with a 490E multiwavelength detector. PTH-amino acids and PTH-glycoamino acids were separated on a MilliGen/BioSearch SequeTag™ (4 μm, 3.9 mm × 300 mm) C_{18} reversed phase column using the manufacturer's recommended gradient program. The two different solvent systems used to examine PTH-amino acid/PTH-glycoamino acid separation are detailed in the Figure legends (Figs 1 and 3).

Analysis of the monosaccharide composition of PTH-glycoamino acid

The PTH-glycoamino acids collected from the MilliGen ProSequencer (approximately 1 nmol) were hydrolysed in 2 M TFA at 100 °C for 4 h. After evaporation of the acid, the liberated monosaccharides were analysed by HPAEC using a CarboPac PA1™ column (4 mm × 250 mm, Dionex Corp., USA) with a Waters 600 LC system and Waters 464 pulsed amperometric electrochemical detector.

Electrospray ionization mass spectrometry of PTH-Asn-494(Sac)

Mass spectra were acquired on a Perkin Elmer/Sciex API III triple quadrupole mass spectrometer (PE/Sciex, Ontario, Canada), equipped with an ionspray atmospheric pressure ionization source. Samples (200 pmol of PTH-Asn(Sac) from cycle 10 of the tryptic glycopeptide) were injected into a moving solvent [10 μl min^{-1}; 50% (v/v) acetonitrile, 0.5% (v/v) TFA], coupled directly to the ionization source via a fused silica capillary interface (50 μm i.d. × 50 cm length). Sample droplets were ionized at a positive potential of 5 kV and entered the analyser through an interface plate and subsequently through an orifice (100–120 μm diameter) at a potential of 80 V (a sufficient potential to induce a limited amount of dissociation within the molecule). Full scan spectra were acquired over the mass range 400–2200 Da with a scan step size of 0.1 Da.

Results and discussion

Desialylation of glycopeptides increases the yield of glycoamino acids recovered during solid-phase Edman degradation

Covalent attachment of a glycopeptide by acid amino acid (Asp, Glu) side chains and C-terminal carboxyls to immobilized amine groups provides a generalized approach to solid-phase sequencing [16]. Even if the peptide of interest lacks amino acid side chain carboxyls (i.e. Asp and Glu), all peptides have a C-terminal carboxyl group. To increase the yield of glycopeptides covalently bound to an arylamine derivatized membrane support, we followed the recommendations of Laursen *et al.* [15] and coupled the glycopeptide at 4 °C. Although the initial yields were encouraging (55%), the corrected yield for Asn(Sac)494 (cycle 10) was disappointing (less than 15% of the expected yield in cycle 10). The low yield of PTH-Asn(Sac) (Figs 1b and 2a) suggested to us that in addition to the amino acid side chain and C-terminal carboxyls, the terminal sialic acid carboxyl groups of the oligosaccharide also formed an amide bond with the arylamine membrane, hence immobilizing the PTH-Asn(Sac) on the PVDF disc. To test this, the tryptic peptide was first subjected to mild acid hydrolysis to remove sialic acids, then subjected to solid-phase Edman degradation following coupling to Sequelon-AA. The initial yield of the desialylated glycopeptide was

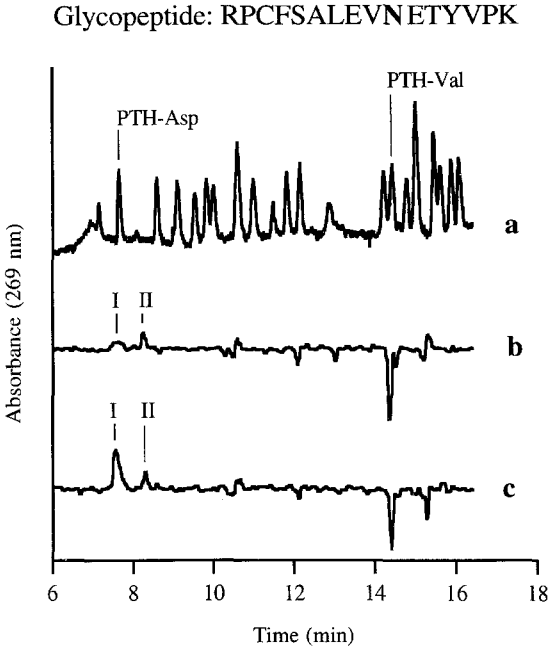

Figure 1. HPLC chromatograms of PTH-amino acid standards and PTH-Asn(Sac) released after solid-phase Edman degradation of the Casebrook tryptic fragment. Chromatography conditions were; solvent A: 35 mM ammonium acetate pH 4.9; solvent B 100% acetonitrile. The flow rate was 0.7 ml min^{-1} and column oven temperature 50 °C. The gradient for PTH-amino acid separation was that recommended by the manufacturer. (a) Thirty-five pmol PTH-amino acid standard chromatogram. The elution position for PTH-Asp and PTH-Val are indicated. Chromatogram for cycle 10 of ≈100 pmol of the sialylated (b) and desialylated (c) albumin tryptic glycopeptide covalently coupled to Sequelon-AA at 4 °C. PTH-Val released at cycle 9 can be seen as the negative peak. The elution position of PTH-Asn(Sac)I is 7.5 min and PTH-Asn(Sac)II is 8.3 min.

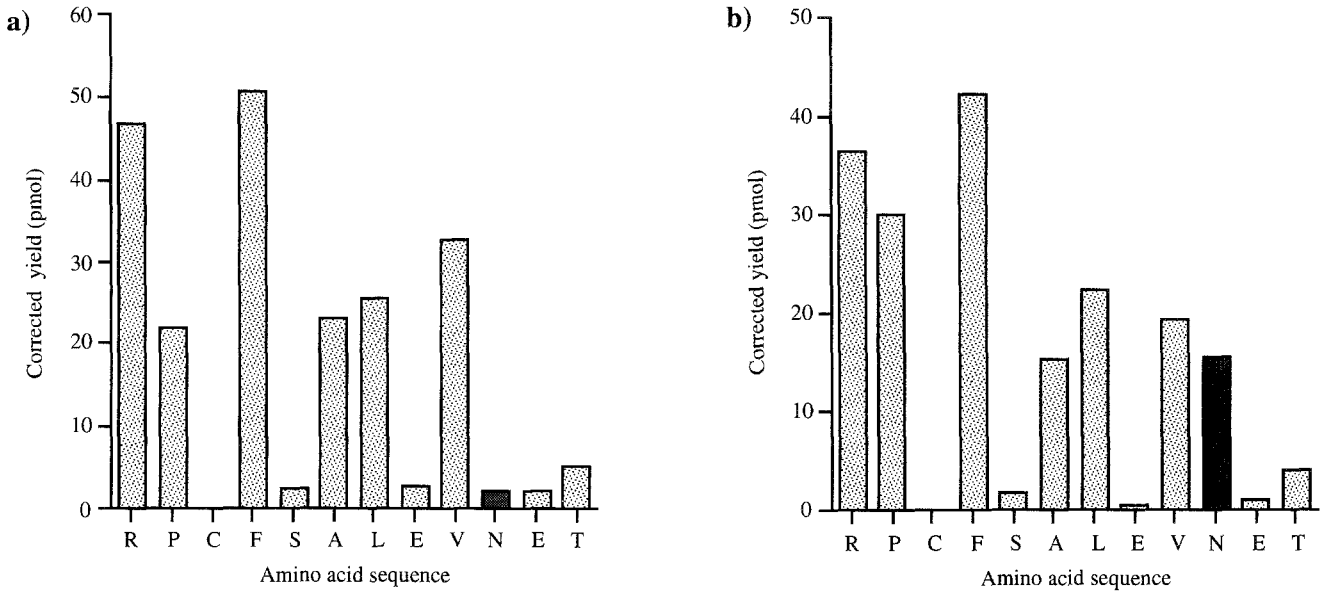

Figure 2. Corrected yields for PTH-amino acids from the solid-phase Edman degradation of the sialylated (a) and desialylated (b) Casebrook tryptic glycopeptide for amino acids Arg485-Thr496. Non-glycosylated amino acids are shown by shaded bars and the glycosylated Asn by a solid bar. Cysteine was reduced and alkylated with AEDANS. The derivatized Cys was recovered in low yield and is not represented in the graph. Glutamic acid was recovered in low yield due to the coupling of the side chain carboxyl to the Sequelon-AA membrane.

slightly less than the sialylated peptide (45% compared with 55%) probably due to loss of peptide during the desialylation step. However, the yield of PTH-Asn(Sac) was significantly increased (Figs 1c and 2b) to approximately 75% of the expected yield in cycle 10, presumably because it was not coupled to the immobilized amine groups.

Purification and characterization of the PTH-glycoamino acid

With conditions optimized for PTH-Asn(Sac) yield, attempts were made to obtain compositional analysis on the recovered oligosaccharide. Following 2 M TFA hydrolysis of PTH-Asn494(Sac), the yields of monosaccharides were dwarfed by a high glucose contamination (data not shown). Glucose is a ubiquitous contaminant, which is difficult to exclude from any compositional assay. The principal source of glucose contamination was localized to the PTH-amino acid analysis buffer, ammonium acetate. Rather than search for a new source of glucose-free ammonium hydroxide/acetic acid, we established an alternative separation system, using a slightly acidic solvent system, either 2 mM acetic acid or 0.001% (v/v) TFA.

One advantage of the new elution conditions, in addition to overcoming the glucose contamination problem, was that there was a better separation of the PTH-Asn(Sac) from the amino acids PTH-Asp and PTH-Asn (Fig. 3). Using the manufacturer's recommended buffer system and gradient, the major PTN-Asn(Sac) peak coelutes with PTH-Asp ([11, 12] and Fig. 1) which could suggest that repeated cycles of Edman degradation deamidate PTH-Asn(Sac) to PTH-Asp. However, under the new chromatographic conditions PTH-Asn(Sac) is clearly resolved from PTH-Asp (Fig. 3), which confirms that the sugar chain remains attached to the amide. An alternative buffer system using triethylamine phosphate has been recently described by Strydom [17] which also provides an early chromatographic space for the elution of hydrophilic post-translationally modified PTH-amino acids. However, we have not yet established whether this buffer system has a low glucose content compatible with monosaccharide compositional analysis.

The fact that the major PTH-Asn(Sac) peak and PTH-Asp coelute using the manufacturer's recommended buffer system has led others [18] to conclude that a covalently bound N-glycosylated peptide from human glucocerebrosidase treated with the endoglycosidase PNGase resulted in deamidation of the PTH-Asn(Sac) to PTH-Asp. However, we believe that Martin and Eliason [18] show that the covalently bound glycopeptide could be resistant to the endoglucosidase; the pattern of peaks recovered for PTH-Asn(Sac) from the glucocerebrosidase are identical to those we recovered for PTH-Asn(Sac) from ovomucoid Asn10(Sac) and Casebrook mutant albumin Asn494(Sac) [11].

The oligosaccharide is degraded slowly during repeated cycles of Edman degradation

After 10 cycles of Edman degradation there is chromatographic evidence for heterogeneity indicative of some degradation (Fig. 1c). The stability of Asn(Sac) to repeated cycles of Edman degradation was therefore examined by sequencing nmol quantities of both the V8 peptide

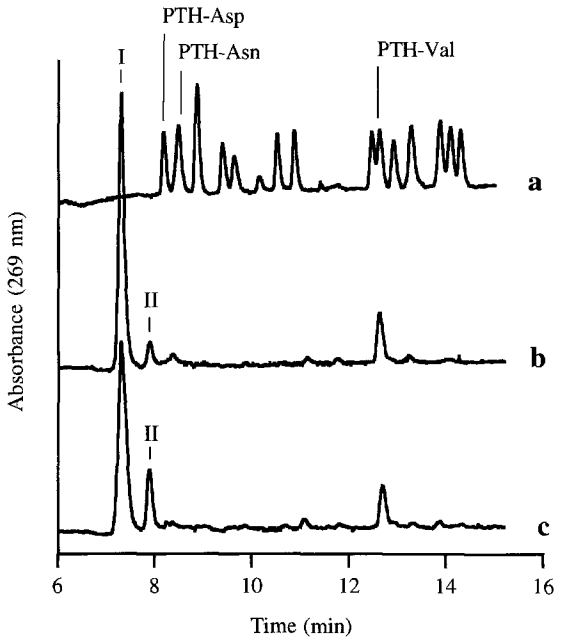

Figure 3. HPLC chromatograms of PTH-amino acid standards and PTH-Asn(Sac) released after solid-phase Edman degradation of the Casebrook tryptic and V8 fragments. Chromatography conditions were; solvent A: 2 mM acetic acid; solvent B 100% acetonitrile. The flow rate was 0.7 ml min^{-1} and column oven temperature 50 °C. The shape of the gradient for PTH-amino acid separation was not altered from the gradient used for the elution of PTH-amino acids with 35 mM ammonium acetate pH 4.9 buffer. (a) 35 pmol PTH-amino acid standard chromatogram. The elution position for PTH-Asp and PTH-Val are indicated. (b) PTH-amino acid chromatogram for cycle 2 of 1.0 nmol of the desialylated albumin V8 glycopeptide (Val493-Glu495) covalently coupled to Sequelon-AA at 4 °C. PTH-Val released at cycle 9 can be seen at approximately 12.5 min. (c) PTH-amino acid chromatogram for cycle 10 of 1.0 nmol of the desialylated albumin tryptic glycopeptide (Arg485-Lys500) covalently coupled to Sequelon-AA at 4 °C.

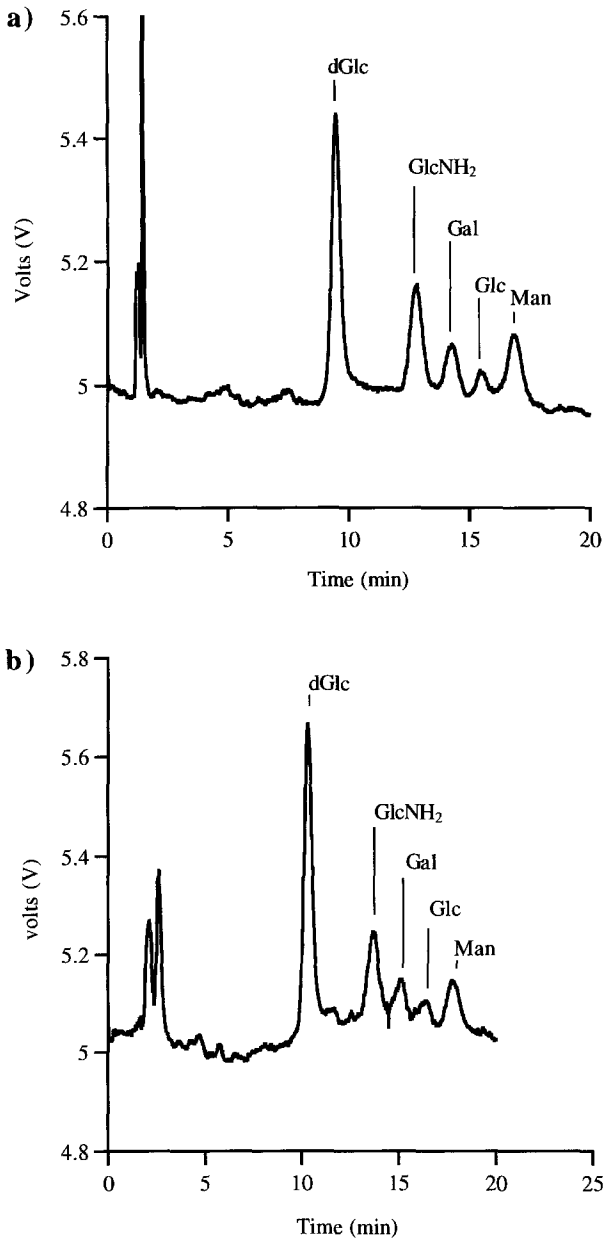

Figure 4. High performance anion exchange chromatograms using pulsed amperometric detection of monosaccharides released by 2 M TFA hydrolysis of peak I of PTH-Asn(Sac). The sugars were eluted isocratically with 15 mM NaOH and post-column addition of 0.4 M NaOH and identified by comparison with standards. An internal standard of 2-deoxyglucose was used for quantitation. $GlcNH_2$ (glucosamine), Gal (galactose), Glc (glucose), Man (mannose). Chromatogram (a) represents the compositional analysis of 360 pmol of PTH-Asn494(Sac) released at cycle 10 of the tryptic peptide, while chromatogram (b) represents the compositional analysis of peak I of 400 pmol of PTH-Asn494(Sac) released at cycle 2 of the V8 peptide.

(Val493-Glu495), where Asn(Sac) appears in cycle 2, and the tryptic peptide (Arg485-Lys500) where Asn(Sac) appears in cycle 10. There is evidence of partial hydrolysis of the oligosaccharide with the increase in yield of PTH-Asn(Sac)II after 10 cycles (to 14% of total yield) compared with PTH-Asn(Sac)II (5% of total yield) in two cycles (Figs 3b,c). However, the combined yields of the major (I) and minor (II) peaks for PTH-Asn(Sac) from cycle 2 (V8 glycopeptide) and cycle 10 (tryptic glycopeptide) were essentially identical.

The major PTH-Asn(Sac) peaks from cycle 2 of the V8 glycopeptide (420 pmol) and cycle 10 of the tryptic glycopeptide (360 pmol) were collected and subjected to compositional analysis by HPAEC-PAD (Fig. 4). Monosaccharide composition was quantified by the inclusion of 1 μg of the internal standard deoxyglucose (Table 1). The observed composition is consistent with the presence of a complex biantennary oligosaccharide; $GlcNAc_4$:Man_3:Gal_2, previously reported for Casebrook albumin [19]. Hence, much of the desialylated oligosaccharide structure remains intact on the glycosylated Asn during as many as 10 cycles of Edman degradation. The decrease in the galactose/

Table 1. Monosaccharide composition of Casebrook PTH-Asn494(Sac).

Sugar constituent	*Composition (mol/mol[a])*	
	Cycle 2[b]	*Cycle 10[c]*
Galactosamine	0	0
Glucosamine	3.8	3.2
Galactose	2.2	1.7
Mannose	2.6	2.9

[a] Normalized on the amount (≈400 pmol) of PTH-Asn(Sac) collected.
[b] Peak I of the PTH-Asn(Sac) recovered from cycle 2 of the V8 glycopeptide.
[c] Peak I of the PTH-Asn(Sac) recovered from cycle 10 of the tryptic glycopeptide.

mannose ratio for PTH-Asn(Sac) from cycle 10 of the tryptic glycopeptide (see Table 1) suggests the loss of a terminal galactose, which is consistent with the asymmetric shape of PTH-Asn(Sac)I (Figs 1c and 3c). PTH-Asn(Sac)II was subjected to compositional analysis by HPAEC-PAD and identified as $GlcNAc_2$:Man_1 (data not shown). We are currently investigating moderated cleavage conditions to limit this complication.

Ionspray MS of PTH-Asn(Sac)

Additional evidence concerning the nature of the oligosaccharide attached to PTH-Asn494 was obtained by ionspray MS. The expected mass for PTH-Asn-$GlcNAc_4$:Man_3:Gal_2 is 1872.8 Da and the observed molecular weight was 1872.9 Da (Fig. 5). Limited structural information was attained by increasing the orifice potential (Fig. 5). However, interpretation of the spectrum is difficult because of the likelihood that some of the fragment ions arose from products of degradation during Edman sequencing.

The method described in this paper makes possible the analysis of the sugars attached to a specific amino acid. The oligosaccharide is released by Edman degradation as a labelled reducing terminal sugar, the PTH-glycoamino acid. This provides an excellent chromophore in the UV range (269 nm max), which should prove useful for the separation of heterogeneous oligosaccharides on a single amino acid. There are several excellent methods for the analysis of *N*-linked glycosylation sites, particularly LC-MS analysis of a glycoprotein proteinase digest and subsequent identification of the glycopeptides by the diagnostic sugar oxonium-ions [6]. However, solid-phase Edman degradation of glycopeptides which contain a domain of clustered glycosylation sites is the method of choice for glycosylation site identification and characterization. Mass spectral analysis alone will not provide specific structural information regarding the composition of hexoses and hexosamines and techniques such as acid hydrolysis of the oligosaccharide and subsequent identification of the sugars by HPAEC or GC-MS is necessary for complete carbohydrate analysis.

Clustered glycosylation sites are typical of *O*-glycosylated proteins such as the mucins and proteoglycans [7]. We have previously demonstrated the efficiency of solid-phase Edman degradation by sequencing through the N-terminal domain of the 'mucin-like' red blood cell glycoprotein GpA; in that study we positively identified 1 *N*-linked and 16 *O*-linked amino acids in the 52 amino acids sequenced [12]. As demonstrated here, solid-phase Edman degradation in combination with techniques of carbohydrate analysis such as HPAEC and MS, will allow a new approach to the

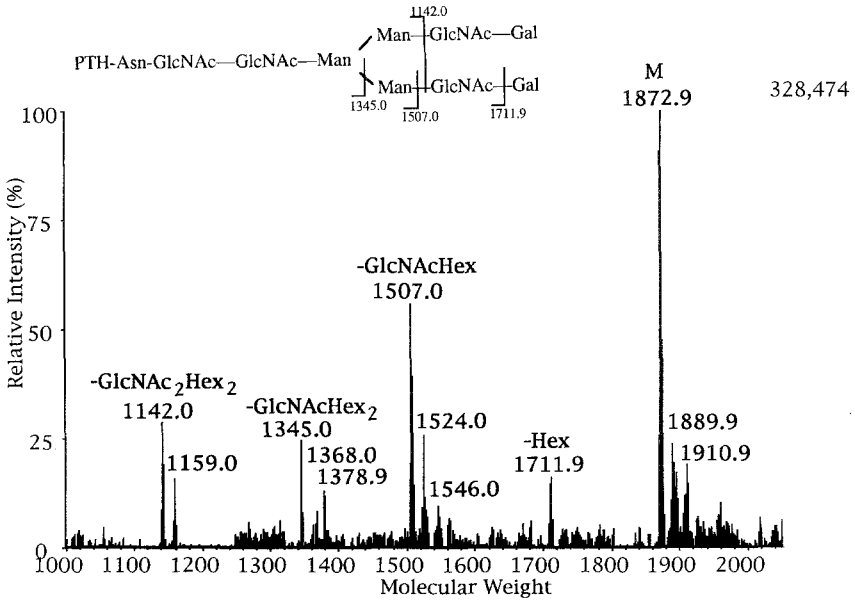

Figure 5. The reconstructed ion-spray spectra for peak I of PTH-Asn(Sac) released at cycle 10 from the tryptic peptide. Mass of PTH-Asn = 249.3.

characterization of heavily glycosylated proteins previously thought intractable for protein chemistry studies.

Acknowledgements

MUCAB research on Glycobiology is supported by an ARC Program grant to K.L.W. and an NH&MRC grant to K.L.W. and A.A.G. We thank Millipore Australia for support of our solid-phase sequencing project.

References

1. Dwek RA, Edge CJ, Harvey DJ, Wormald MR, Parekh RB (1993) *Ann Rev Biochem* **62**:65–100.
2. Hardy MR, Townsend RR, Lee YC (1988) *Anal Biochem* **170**:54–62.
3. Townsend RR, Hardy MR, Lee, YC (1989) *Methods Enzymol* **179**:65–76.
4. Townsend RR, Hardy MR (1991) *Glycobiology* **1**:139–47.
5. Jackson, P (1994) *Anal Biochem* **216**:243–52.
6. Carr SA, Huddleston MJ, Bean MF (1993) *Prot Sci* **2**:183–96.
7. Carraway KL, Hull SR (1991) *Glycobiology* **1**:131–8.
8. Classon BJ, Brown MH, Garnett D, Somoza C, Barclay AN, Willis AC, Williams AF (1992) *Int Immunol* **4**:215–25.
9. Baenziger J, Kornfeld S (1974) *J Biol Chem* **249**:7270–81.
10. Fiat A-M, Jollês J, Aubert J-P, Loucheux-Lefebvre M-H, Jollês P (1980) *Eur J Biochem* **111**:333–9.
11. Gooley AA, Classon BJ, Marschalek R, Williams KL (1991) *Biochem Biophys Res Commun* **178**:1194–1201.
12. Pisano A, Redmond JW, Williams KL, Gooley AA (1993) *Glycobiology* **3**:429–35.
13. Peach RJ, Brennan SO (1991) *Biochim Biophys Acta* **1097**:49–54.
14. Gorman JJ (1987) *Anal Biochem* **160**:376–87.
15. Laursen RA, Lee TT, Dixon JD, Liang S-P (1991) In *Methods in Protein Sequence Analysis* pp. 47–54 (Jornvall H, Hoog J-O and Gustvasson A-M, eds). Birkhauser Verlag, Switzerland.
16. Coull JM, Pappin DJC, Mark J, Aebersold R, Koester H (1991) *Anal Biochem* **194**:110–20.
17. Strydom DJ (1994) *J Chromatogr* **662**:227–33.
18. Martin BM, Eliason WK (1991) In *Techniques in Protein Chemistry II* (Villafranca, JJ ed.) pp. 191–6. San Diego: Academic Press.
19. Haynes PA, Batley M, Peach RJ, Brennan SO, Redmond JW (1992) *J Chrom Biomed App* **581**:187–93.