Glycoconjugate Journal (1994) **11**:187–193

Analysis of glycosylphosphatidylinositol membrane anchors by electrospray ionization-mass spectrometry and collision induced dissociation

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Received 7 April 1994, revised 2 June 1994

The multi-component nature of glycosylphosphatidylinositol membrane anchors makes the analysis of their structure complex. Nuclear magnetic resonance spectroscopy of delipidated glycosylphosphatidylinositol-peptide fractions can supply considerable information but requires relatively large quantities of material. High-sensitivity sequencing techniques are available for the oligosaccharide portions of glycosylphosphatidylinositol anchors, but there is no simple and generally applicable technique to complement this information. In this paper we describe the application of electrospray ionization-mass spectrometry and collision induced dissociation to study intact glycosylphosphatidylinositol-peptides from a *Trypanosoma brucei* variant surface glycoprotein. Collision of the $[M + 4H]^{4+}$ pseudomolecular ions of two glycosylphosphatidylinositol-peptide glycoforms produced easily interpretable daughter ion spectra, from which detailed information on the lipid moiety, carbohydrate sequence and site of peptide attachment could be obtained. All of the collision induced dissociation cleavage events occurred in the glycosylphosphatidylinositol portion of the glycosylphosphatidylinositol-peptide. This technique supplies complementary data to the high-sensitivity oligosaccharide sequencing procedures and should greatly assist glycosylphosphatidylinositol anchor structure-function studies. particularly when sample quantities are limiting.

Keywords: glycosylphosphatidylinositol; electrospray; mass spectrometry; collision induced dissociation; Trypanosoma

Introduction

Glycosylphosphatidylinositol (GPI) anchors are found on a wide variety of eukaryote cell-surface proteins. In mammalian cells they have been associated with functions such as intracellular targeting, potocytosis and signal transduction. In addition, GPI-anchored proteins and GPI-related glycophospholipids have been implicated in the survival and infectivity of several protozoan parasites. The distribution, structure, biosynthesis and function of GPI molecules have been extensively reviewed [1–6].

Protein-linked GPI anchors have a conserved core structure of: protein-ethanolamine-PO_4-Manα1-2Manα1-6Manα1-4Glc$NH_2$1-6*myo*-inositol-1-PO_4-lipid. However, many anchors have additional residues attached to this conserved structure. Mammalian, fish and *Dictyostelium* GPI anchors have one or two additional ethanolamine phosphates attached to the glycan core [7–14]. The cores are also substituted by sugars in the anchors of proteins from rat brain [7], hamster brain [15], pig and human kidney [16], *Trypanosoma cruzi* [17], *Torpedo* [13], *Dictyostelium* [14] and *Saccharomyces cerevisiae* [18]. In the case of *Trypanosoma brucei*, the procyclic acidic repetitive protein has *N*-acetylglucosamine, galactose and sialic acid substitutions [19], while the anchor of the variant surface glycoprotein (VSG) coat, from the bloodstream form of the parasite, has a branched α-galactose side chain [20]. The lipid moiety attached to the inositol ring through a phosphodiester linkage is also quite variable, reviewed recently in [6]. The GPI anchors of *Saccharomyces cerevisiae* and *Dictyostelium discoideum* contain ceramides, but glycerolipids are more common. Alkyl-acyl-glycerols, diacyl-glycerols and *lyso*-acyl-glycerols have all been reported and some GPI anchors contain an additional fatty acid (palmitate) in hydroxy-ester linkage to the inositol ring [21, 22].

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Two-dimensional NMR has been an invaluable tool in solving the structures of a variety of GPI anchors and related glycophospholipids [7, 13, 18, 20, 23–30]. However, such analyses require relatively large quantities of material. Therefore, when amounts of starting material are limited, only combinations of the other complementary methods can be used. These include exoglycosidase micro-sequencing [7, 13, 14, 18, 20, 25, 31–36] and fast-atom bombardment mass spectrometry of (mostly) modified and/or derivatized material [8, 15, 18, 26–28, 37–43]. More recently, electrospray mass spectrometry has been applied to GPI anchors [10, 15, 44, 45].

Here we report on the use of electrospray-mass spectrometry (ES-MS), in association with collision induced dissociation (CID), to give structural detail on an unmodified GPI-peptide fragment isolated from a *Trypanosoma brucei* variant surface glycoprotein (VSG).

Materials and methods

Purification of mfVSG117

Trypanosoma brucei bloodstream trypomastigotes (variant MITat 1.4, also known as variant 117) were purified from infected rat blood as previously described by Cross [46]. Biosynthetic labelling of 3.5×10^9 cells with [^{3}H]myristate was performed as described by Ferguson and Cross [47]. The labelled cells were washed once in cold medium and added to unlabelled cells (2×10^{10}). The mixed cells were washed again with cold medium and membrane form VSG (mfVSG117) was purified from the resulting cell pellet according to the method of Hereld *et al.* [48]. Analysis of this material by SDS-PAGE revealed a substantially pure preparation of mfVSG (data not shown).

Purification of the GPI-peptide

The mfVSG (200 nmol, as measured by *myo*-inositol content) was resuspended in 7.8 ml 0.1% Triton X-100, containing 100 mM NH_4HCO_3. Pronase (0.2 mg) was added in 200 µl of 10 mM calcium acetate. After 18 h at 37 °C a further aliquot of 0.2 mg Pronase was added and digestion was continued for another 10 h. The digest was extracted four times with 2 ml toluene, freeze dried and resuspended in 100 µl water. The suspension was extracted three times with an equal volume of 1-butanol saturated with water. The aqueous phase was dried, resuspended in 1 ml 100 mM ammonium acetate, 5% (v/v) 1-propanol and centrifuged to remove insoluble material. The supernatant was applied to a column of octyl-Sepharose (10 × 1 cm) and allowed to interact with the gel for 1 h. The column was then eluted with a linear gradient from 100 mM ammonium acetate, 5% (v/v) 1-propanol to 60% 1-propanol over 200 min at 0.5 ml min^{-1} and 1 ml fractions were collected. The GPI-peptide was located by liquid scintillation counting of aliquots of the fractions, and by orcinol-staining of aliquots spotted on to a thin layer chromatography plate. The 1-propanol concentrations were measured by refractive index. Peak fractions of coincident [^{3}H]myristate label and orcinol staining (which eluted at about 40% 1-propanol) were pooled and the *myo*-inositol content was measured by GC-MS. The yield of purified GPI-peptide was 72%. The pooled fractions were dried and stored at −20 °C.

Electrospray mass spectrometry

Mass spectrometric data were acquired on a VG Quattro-BQ (Fisons Instruments, VG Biotech, Altrincham, UK), a triple quadrupole instrument with a mass range for singly charged ions of 4000. Mass spectra were recorded by linear scanning over the range *m/z* 900–1900 in 10 s at an instrument resolution of 2000 (using the full width at half-maximum definition). Several scans were summed from a 10 µl introduction of sample solution (20 pmol µl^{-1}) in acetonitrile, water (1:1, v/v) containing 0.2% formic acid. The flow rate of sample to the electrospray source was 4 µl min^{-1}. The mass spectra, containing multiply-charged ions, were recorded on an *m/z* scale and were transformed on to a true molecular mass scale using the software supplied with the instrument. Mass-scale calibration employed the multiply charged ion series from a separate introduction of bovine ubiquitin (Sigma Cat. No. U-6253, average molecular mass 8564.9).

Fragment ion spectra generated from the quadruply-charged protonated pseudomolecular ions were obtained by collision induced dissociation in the collision cell using argon as the collision gas at a pressure of 10^{-2} mbar. The sample (50 pmol µl^{-1}) was introduced as described above. The precursor ions were accelerated into the collision cell through a potential difference of 15 V. The CID mass spectra were recorded by linear scanning over the range *m/z* 50–1900 in 10 s. In this case the resolution of the quadrupoles was reduced to about 300 in order to increase the sensitivity. Two 10 µl introductions of the sample solution described above were employed to acquire the spectra. Calibration of the *m/z* scale was by means of the spectrum produced by a mixture of polypropylene glycols 425, 1000 and 2000 (Aldrich Chemical Co. Ltd) at concentrations of 0.1, 0.2 and 0.5 µg µl^{-1} respectively in acetonitrile, water (1:1, v/v) containing 2 mM ammonium acetate.

Results

Heterogeneity of the GPI-peptide

The spectrum shown in Fig. 1a was obtained from the ES-MS analysis of the *T. brucei* VSG117 GPI-peptide. The major ions, at *m/z* 1237.6, 1278.3 and 1318.8, correspond to $[M + 4H]^{4+}$ pseudomolecular ions which, on transformation, yield molecular masses of 4947.0, 5109.3 and 5271.3 Da, respectively (Fig. 1b). The transformed data also reveal 3 additional GPI-peptide species with molecular

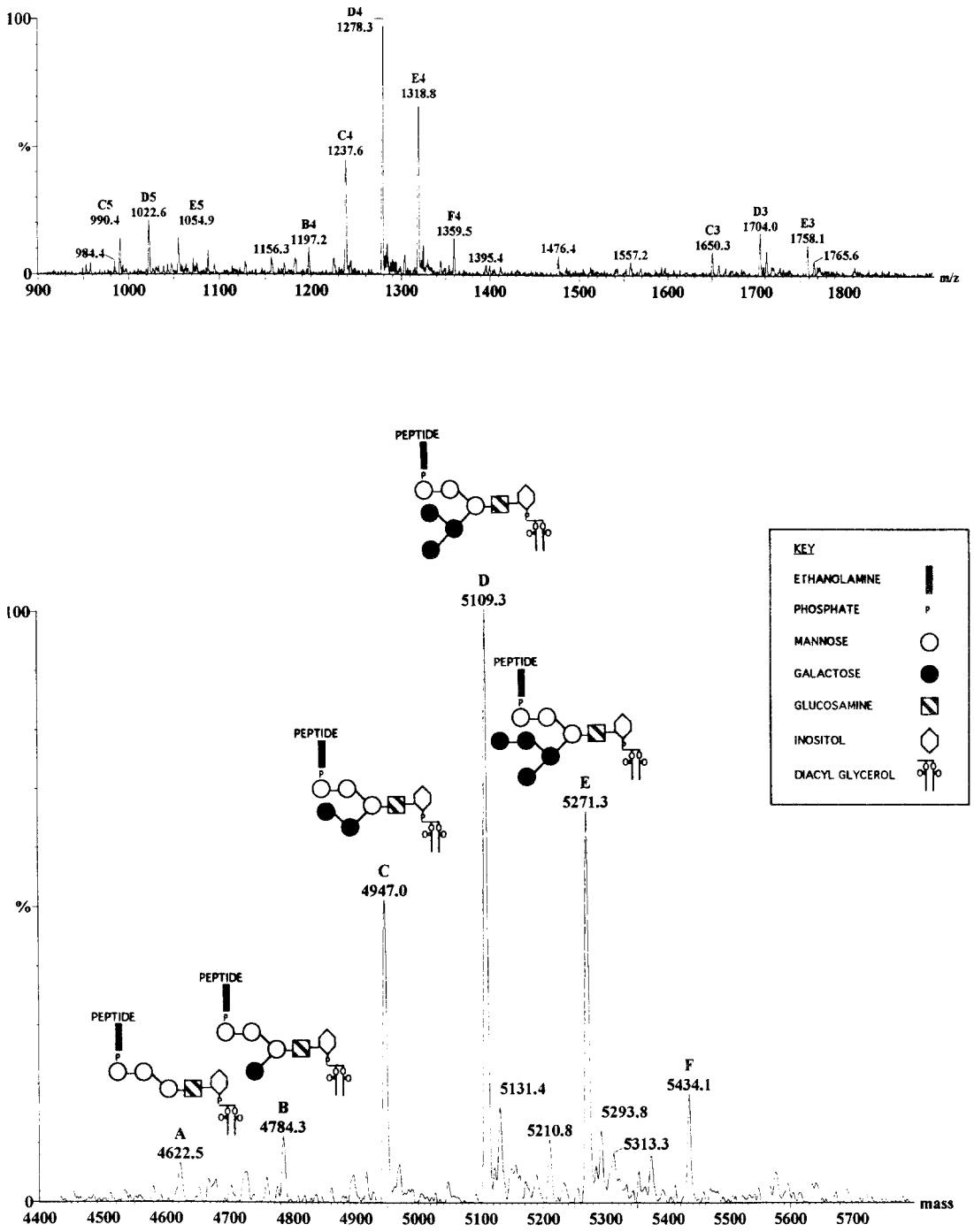

Figure 1. Positive ion electrospray mass spectrum of GPI-peptides from *T. brucei* VSG. The raw (panel a top) and transformed (panel b bottom) mass spectrum of the mixed glycoforms of *T. brucei* VSG GPI-peptide. The species A–F correspond to different levels of galactosylation. The structure corresponding to the cartoon for species E is [Thr_{444}-Asp_{470}]-ethanolamine-HPO_4-6Manα1-2Manα1-6((Galα1-2Galα-6)(Galα1-2)Galα1-3)Manα1-4GlcNH_2α1-6*myo*-inositol-1-HPO_4-dimyristoylglycerol. The peptide sequence is TDKCK-GKLEDTCKKESTCKWEDNACKD, with disulphide bridges between the first and third and second and fourth Cys residues.

masses of 4622.5, 4784.3 and 5434.1 Da (Fig. 1b). Mass heterogeneity between these major peaks is limited to intervals of 162 Da. This suggests that the major structural heterogeneity in the GPI-peptide fraction resides in the number of hexose units and, correspondingly, that the lipid and peptide moieties must be essentially homogeneous*. Taking into account the masses of the known GPI-anchor

* The presence of the small peak of mass 5210.8 (Fig. 1b) suggests that some of the GPI peptides contain an additional Thr residue, i.e. that they contain the peptide Thr_{443}-Asp_{470}

Table 1. Comparison of the calculated (M_rc) and measured (M_rm) molecular mass for each glycoform of the GPI-peptide.

Peak	$(Gal)_n$	$M_r c$	$M_r m$	% *Accuracy*	% *Abundance*
A	0	4623.1	4622.5	0.01	2.5
B	1	4785.2	4784.3	0.02	4.3
C	2	4947.3	4947.0	0.01	19.9
D	3	5109.5	5109.3	0.01	39.1
E	4	5271.6	5271.3	0.01	25.7
F	5	5433.8	5434.1	0.01	8.5

structures [20] and the VSG COOH-terminal peptide sequence, including the disulphide bonds [49–51], the molecular mass data presented in Fig. 1b reveal a peptide component of Thr_{444}-Asp_{470}, where the GPI-anchor is attached to Asp_{470}. Comparison of the predicted molecular masses of the GPI-peptide glycoforms with the molecular masses from ES-MS (Table 1) show the high mass accuracy obtained using this technique. The relative abundances of the major ions C, D and E (Fig. 1b and Table 1) suggest a ratio of the Gal_2 to Gal_3 to Gal_4 GPI-peptide species of 1:2:1.3. These figures are in reasonable agreement with the figures of 1:2:1.7 previously estimated [20].

It is of interest to note that 27 amino acids remained attached to the GPI-anchor following Pronase digestion of membrane form VSG117. This is in contrast to Pronase digestion of soluble form VSG117 (lacking the diacylglycerol lipid moiety) which produces a COOH-terminal fragment containing only Asp_{470} [52]. This suggests that the presence of the anchor may protect the peptide portion of mfVSG from complete proteolytic digestion, probably due to the micellar nature of the GPI-peptide.

While the mass accuracy obtained from the ES-MS spectrum was sufficient to account for all the known components of the various GPI-peptide species, no detailed structural information could be obtained from these data.

Collision induced dissociation analysis

Collision induced dissociation mass spectrometry-mass spectrometry (MS-MS) was performed on the two most intense pseudomolecular ions at *m/z* 1278.3 and 1318.8 (see Fig. 1a), which correspond to $[M + 4H]^{4+}$ pseudomolecular ions for the Gal_3 and Gal_4 GPI-peptide species respectively. The ions of interest were selected in the first quadrupole, collided with argon in the second quadrupole and the resulting daughter ions were analysed in the third quadrupole.

The CID mass spectra obtained on collision of the pseudomolecular ion of the Gal_3 (Fig. 2a) and Gal_4 (Fig. 2b) GPI-peptides reveal that fragmentation occurs preferentially within the GPI-anchor portion. The interpretation described below refers to the spectrum for the Gal_3 GPI-peptide species shown in Fig. 2a. The spectrum for the Gal_4 species is similar and contains four additional ions at *m/z* 1318.8 (a 4^+ ion) and at *m/z* 1506.4, 1592.8 and 1703.7 (3^+ ions), arising from the presence of the extra Gal residue (compare Fig. 2a and Fig. 2b).

The triply-charged (3^+) daughter ions, at *m/z* 1649.3 and 1594.4, and quadruply-charged (4^+) daughter ions, at *m/z* 1237.4, 1197.5 and 1157.7, result from the loss of one or more side-chain hexose (Gal) residues (Fig. 3). Some of the 2^+ and 3^+ daughter ions supply useful sequence information: the 2^+ ions at *m/z* 1547.7, 1570.1, 1611.8, 1690.9 and 1772.6, and the 3^+ ions at *m/z* 1033.0, 1048.0, 1073.1, 1128.4 and 1182.6, are consistent with the sequence: peptide-ethanolamine-HPO_4-Hex-Hex*. The 3^+ ion at *m/z* 1452.0 results from the loss of phosphatidylinositol (PI), while the 3^+ double-cleavage ions at *m/z* 1398.2 and 1344.4 (resulting from the loss of PI *and* 1 and 2 Gal residues, respectively) indicate that the side-chain of three Gal residues is attached to one of the underlined residues in parentheses in the sequence: peptide-ethanolamine-HPO_4-Hex-Hex-[*Hex-HexN*]†. The 3^+ ion at *m/z* 1538.5 results from the loss of dimyristoylglycerol, while the 3^+ double-cleavage ions at *m/z* 1484.7, 1431.1 and 1377.6 correspond to the loss of dimyristotylglycerol *and* 1 to 3 Gal residues. The intense ion at 496.9 (Fig. 2a, inset) corresponds to the [M + H]† dimyristoylglycerol fragment ion.

Preliminary data on this sample have been described [44]. In that study the 4^+ ion for the Gal_3-containing species was subjected to more energetic CID than reported here, leading to greater fragmentation and the generation of considerably more double-cleavage ions. However, the number of easily interpretable ions was about the same as reported here.

Discussion

In complex carbohydrate analysis FAB-MS of native and permethylated and/or peracetylated oligosaccharides is a particularly powerful approach, reviewed in [53–55]. The Pronase-derived, base-hydrolysed, *N*-methylated GPI-peptide of human acetylcholinesterase was successfully studied by positive-ion and negative-ion FAB-MS [8]; permethylated GPI glycan fragments were analysed by FAB-MS-MS [15, 39] and permethylated delipidated GPI-peptides have been analysed by FAB-MS [18]. In addition, the phosphatidylinositol and/or glycerolipid moieties of certain GPI anchors have been analysed by FAB-MS following their release by nitrous acid deamination or phospholipase C digestion [8, 61]. However, native

* The 2^+ and 3^+ ions at *m/z* 1547.7 and 1033.0, respectively, correspond to the peptide component Thr_{444}-Asp_{470} alone (mass accuracy 0.08%).
† There is insufficient mass-resolution formally to distinguish hexosamine from hexose residues. One of the residues is assigned as a hexosamine because all GPI structures contain a $GlcNH_2$ residue adjacent to the PI moiety.

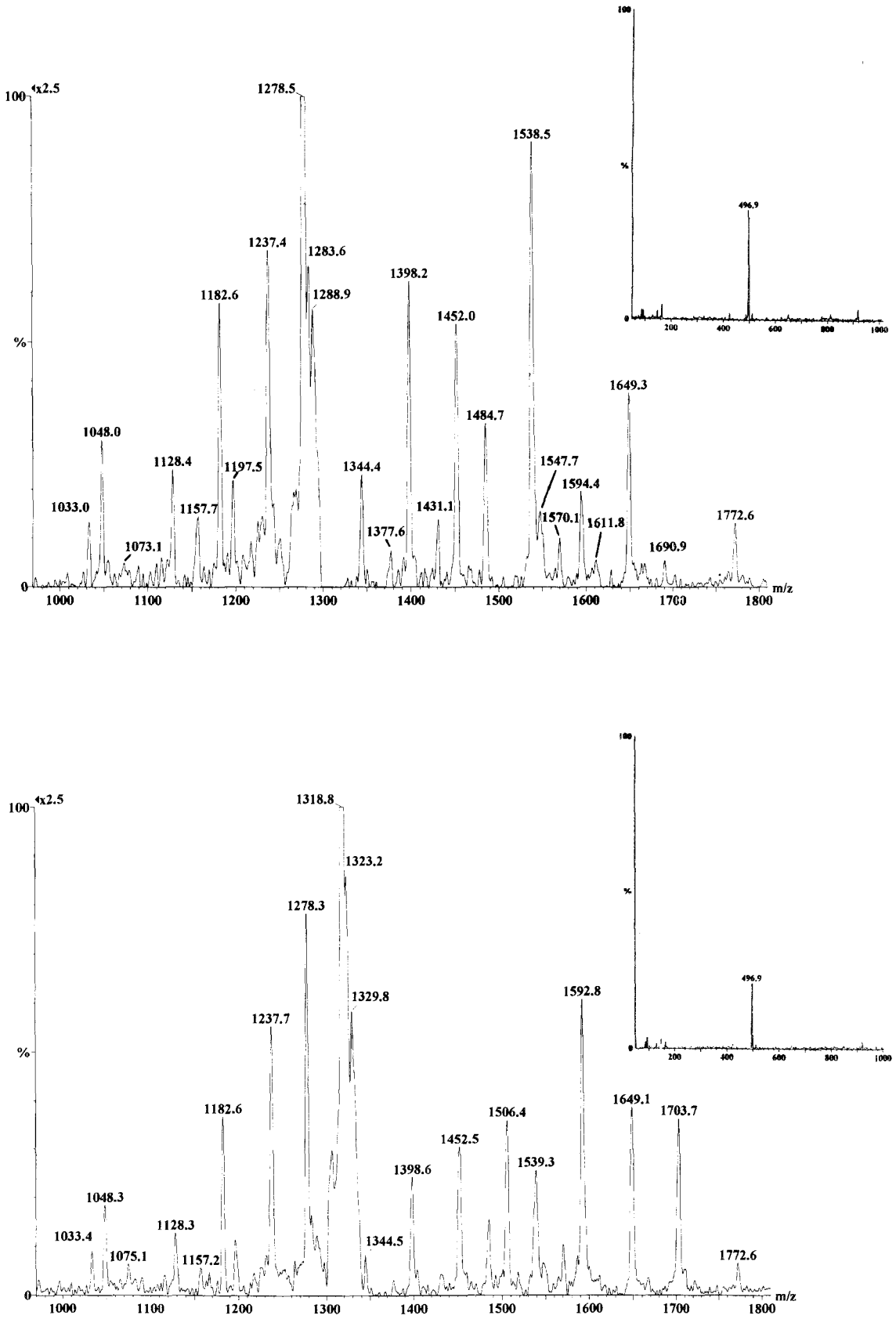

Figure 2. Positive ion tandem mass spectra (MS-CID-MS) of GPI-peptide glycoforms. The $[M + 4H]^{4+}$ ions of the Gal_3 and Gal_4 GPI-peptide species were subjected to CID and the daughter ion spectra were recorded (panels a (top) and b (bottom), respectively). The insets show the low-mass end of the spectra which contain the dimyristoylglycerol fragment ion at *m/z* 496.9.

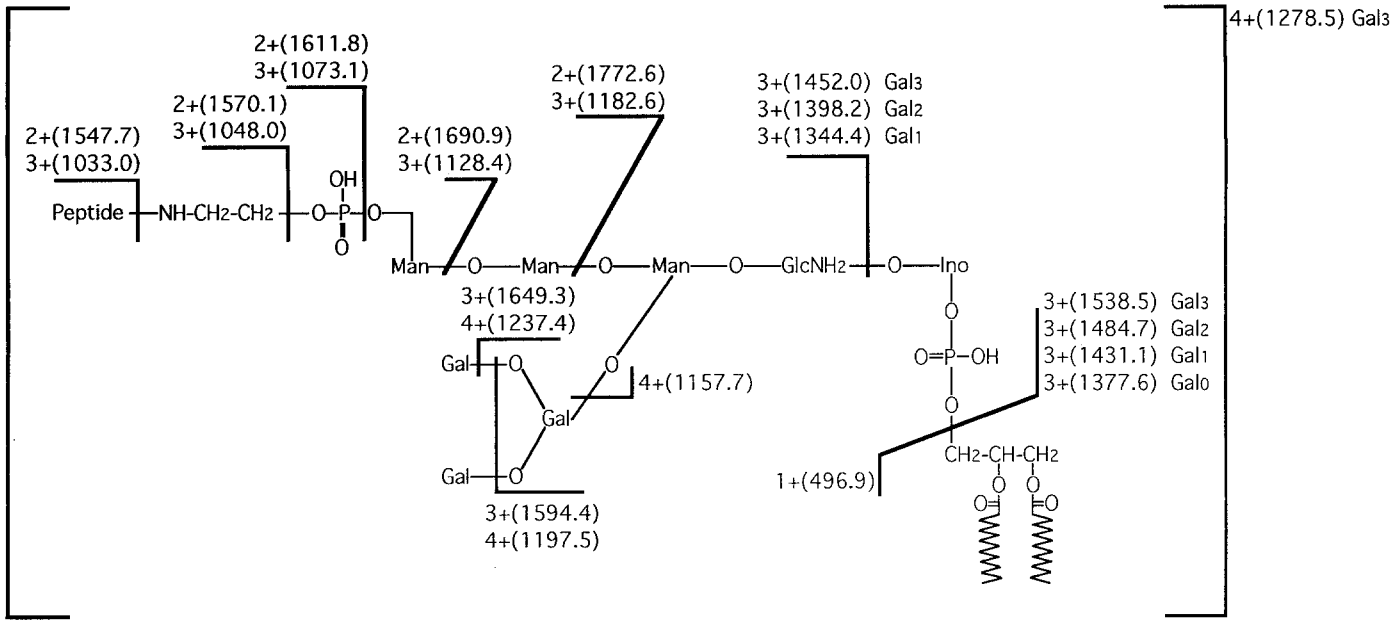

Figure 3. Interpretation of the GPI-peptide fragmentation data. All of the ions labelled in Fig. 2a are assigned. The numbers in brackets refer to the ion *m/z* values, the preceding numbers indicate the number of positive charges and, where relevant, the number of Gal residues left on the side-chain are indicated following the *m/z* value.

GPI-peptides [44] and delipidated GPI-peptide samples [10, 15, 45, 57] have only been successfully analysed by ES-MS. The ability of the native peptide component to acquire multiple positive charges during the electrospray ionization process makes ES-MS an ideal technique for GPI-peptide samples. Furthermore, the predisposition of the GPI portion to undergo collision induced fragmentation, in preference to the peptide portion, makes electrospray-mass spectrometry-mass spectrometry (ES-MS-MS) data easy to interpret with regard to the GPI carbohydrate, lipid and substituent structure.

The ES-MS-MS data alone define the sequence: peptide-ethanolamine - HPO_4 - Hex - Hex - (Hex_3)[Hex, $HexNH_2$]-[Ino, HPO_4]-dimyristoylglycerol. Previously the assignment of the site of peptide linkage to GPI anchors has been particularly difficult [18] but the ES-MS-MS data now makes this easy. Taking into account the conserved Manα1-4$GlcNH_2$α1-6*myo*-inositol-1-HPO_4-lipid motif, found in all GPI structures [6], the sequence can be refined to: peptide - ethanolamine - HPO_4 - Hex - Hex - (Hex_3)[Man-$GlcNH_2$]-Ino-HPO_4-dimyristoylglycerol. This is a considerable amount of structural information from a very small amount of sample (1 nmol of total GPI-peptide, i.e. < 500 pmol each of the Gal_3 and Gal_4 GPI-peptides). Moreover, since these data were acquired, improvements in the instrumentation have increased the sensitivity by a factor of ten. The procedure does not require prior-derivatisation of the sample or prior separation of the individual glycoforms to produce easily interpretable CID fragmentation data.

Like most other mass-spectrometric techniques, the data do not provide any indication of hexose identity, conformation, configuration, linkage composition or anomericity. However, sensitive micro-sequencing procedures for the carbohydrate component alone are already available [31, 32, 34]. The exoglycosidase micro-sequencing of the glycan component of the Gal_3 glycoform of this *T. brucei* VSG anchor has already been reported [34], and from this the glycan has been defined as Manα1-2Manα1-6(αGal_3)Manα1-$GlcNH_2$. Methylation analysis of this same glycan [20] allows this sequence to be further refined to: Manα1-2Manα1-6(Galα1-6(Galα1-2)Galα1-3)Manα1-4$GlcNH_2$. Thus exoglycosidase micro-sequencing and methylation linkage analysis, together with the type of ES-MS-MS analysis of intact GPI-peptides described here, can supply the detailed structure of small quantities (as little as 2 nmol) of GPI-anchor.

Acknowledgements

This work was supported by grants from the MRC and The Wellcome Trust. M.A.J.F. is a Howard Hughes International Research Fellow. In the initial phase of this study, J.E.T.-O. was a Beit Memorial Research Fellow in the Department of Biochemistry, University of Dundee. We thank Angela Mehlert for her assistance with the trypanosome preparations.

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