Trypanosoma brucei brucei variant surface glycoprotein contains non-*N*-acetylated glucosamine

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The C-terminal amino acid of the variant surface glycoprotein of the parasitic protozoan *Trypanosoma brucei brucei* is glycosylated and the oligosaccharide has been shown to contain glucosamine. By acid hydrolysis, HNO₂ deamination and ¹H-n.m.r. studies we have demonstrated that the amino group of this glucosamine is not *N*-acetylated and is most probably unmodified.

INTRODUCTION

Trypanosoma brucei is a parasitic protozoan causing African trypanosomiasis, a disease known as sleeping sickness in man. It evades its mammalian host's immune response by antigenic variation, which is the sequential expression of immunologically distinct variant surface glycoproteins (VSGs) on its surface. The VSGs can be isolated in two forms, the membrane form (mfVSG) and a soluble form (sVSG) (Cardoso de Almeida & Turner, 1983). The mfVSG, prepared by the solubilization of the trypanosomes in boiling detergent, contains glycosyl-sn-1,2-dimyristoyl phosphatidylinositol (Ferguson *et al.*, 1985), whereas the soluble VSG, prepared by ionic disruption of the cells, lacks the sn-1,2-dimyristoylglycerol component.

In all VSGs examined, the C-terminal amino acid is either an aspartic acid or a serine residue (only one exception is known, a glycoprotein having asparagine in this position) (reviewed by Holder, 1985). This residue is amide-linked through its α -carboxy group to ethanolamine, which is in turn *O*-linked to an oligosaccharide (Holder, 1983*a*). The monosaccharide composition of the C-terminal oligosaccharide includes mannose, galactose and glucosamine. Upon acid hydrolysis the glucosamine is recovered in low yield (A.-M. Strang, unpublished work), less than 1 mol/mol of C-terminal amino acid. The stability of its glycosidic linkage is shown here to be due to the presence of a free amino group at the C-2 position of the glucosamine. Non-*N*-acetylated glucosamine has not been previously reported in glycoproteins from eukaryotic cells, with the exception of the heparin and heparan sulphate-containing proteoglycans (Rodén, 1980). In the peptidoglycan of *Bacillus cereus* (a prokaryote), *N*-non-substituted glucosamine residues have also been demonstrated (Araki *et al.*, 1972). Its presence in the VSG is another example of the unusual nature of trypanosome glycoproteins. A preliminary communication on the subject of this paper has been published (Strang *et al.*, 1985).

MATERIALS AND METHODS

Preparation of sVSG and its glycopeptides

Trypanosomes of *T. b. brucei* strain 427 clone 121 (MITat 1.6) were used to prepare sVSG 121 as described by Cross (1975, 1984). Essentially the procedure involved harvesting trypanosomes from infected rat blood, lysis of the cells in 0.2 mM-zinc acetate at 4 °C and incubation of the membranes in 0.01 M-sodium phosphate buffer, pH 8.0, at 45 °C. The released VSG was then purified by ultracentrifugation and ion-exchange chromatography (Cross, 1975, 1984).

The glycopeptides were prepared by Pronase (a proteinase from *Streptomyces griseus*; Sigma Chemical Co., Poole, Dorset, U.K.) digestion of the VSG in 0.1 M-NH₄HCO₃ at 37 °C for 65 h. The digestion products were passed through a 2 ml column of Aminex AG 50W-X4 resin (Bio-Rad Laboratories, Watford, Herts., U.K.) in 0.05 M-pyridine/5.25 M-acetic acid, pH 2.5, and desalted by gel filtration. Two glycopeptides (Holder & Cross, 1981) were obtained, one arising from the glycosylation of an internal asparagine residue and one from the glycosylation of the C-terminal amino acid. These glycopeptides were purified and separated from each other by ion-exchange chromatography on DEAE-cellulose (Whatman DE52; Whatman, Maidstone, Kent, U.K.) using a delayed linear ionic-strength gradient of 0.01–0.50 M-NH₄HCO₃ (Holder & Cross, 1981). The internal glycopeptide is not bound and is eluted in the 0.01 M-NH₄HCO₃, whereas the C-terminal glycopeptide binds to the DEAE-cellulose and is eluted with the gradient. The purity of the glycopeptides was checked by t.l.c. [solvent system: butan-1-ol/acetic acid/water/pyridine (15:3:12:10, by vol.)]. This C-terminal glycopeptide preparation was used for the structural studies.

Amino acid, hexosamine and ethanolamine determination

Samples were hydrolysed in 200 μ l of 3 M-*p*-toluene-sulphonic acid under N₂ at 100 °C for 24 h with

Abbreviations used: VSG, variant surface glycoprotein; sVSG, soluble VSG; mfVSG, membrane-form VSG; Me₃Si, trimethylsilyl.

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p-fluorophenylalanine as an internal standard, and analysed by using a 25 cm column in a Locarte Mini amino acid analyser as described previously (Allen *et al.*, 1976). The program for elution was: 0.2 M-citrate, pH 2.80, 150 min, 50 °C; 0.2 M-citrate, pH 4.25, 200 min, 50 °C; and 0.35 M-citrate, pH 5.28, 20 min, 60 °C.

G.l.c.

Samples, with mannitol as internal standard, were hydrolysed in 500 μ l of 1 M-HCl in methanol under N₂ at 85 °C for 4 h. After neutralization and acetylation (Kamerling *et al.*, 1975), the methyl glycosides were extracted into methanol.

Before examination of a methanol lysate, a portion of the methanolic solution was dried under N₂ at 55 °C and allowed to react with Sil A (Sigma Chemical Co.) to give the Me₃Si derivatives of the monosaccharides.

Samples were examined by using a Perkin-Elmer F33 gas chromatograph fitted with a Chromapak glass-lined metal column, 1.83 m $\times$ 0.3175 cm, packed with 3.5% SE30 on Diatomite C (AWDMCS-treated, 80–100 mesh). They were eluted with N₂ at 15 ml/min by using a temperature program of 130–210 °C at 1° or 1.5°/min (injector/detector temperature at 225 °C). The Me₃Si derivatives were detected by flame ionization.

HNO₂ deamination, pH 3.5

Samples were dried and redissolved in 100 μ l of 0.05 M-sodium acetate/acetic acid, pH 3.5. A 100 μ l portion of 2% (w/v) NaNO₂ (freshly prepared) was added and the mixture was left for 5 h at 24 °C. The products of this deamination were reduced by the addition of 200 μ l of 0.2 M-H₃BO₃, followed by 400 μ l of 0.13 M-NaBH₄ in 0.1 M-NaOH (freshly prepared) and left for 16 h at 24 °C. Acetic acid (10 μ l) was added to the reaction products and the pH checked before removal of cations on a 5 ml column of Dowex 50 resin (X12, H⁺ form, 50–100 mesh) eluted with water. The eluate was collected and freeze-dried. The freeze-dried products were transferred to a hydrolysis tube and repeatedly dried from methanol to remove borate. Methanolysis and preparation for g.l.c. were performed as described above.

HNO₂ deamination, pH 1.5

Samples were dried and redissolved in 20 μ l of water at 0 °C. HNO₂ (80 μ l) freshly prepared at 0 °C by a

method based on that of Shively & Conrad (1976) was added and the mixture was adjusted to 24 °C and left for 10 min. The reaction mixture was dried under N₂ and excess HNO₂ was eliminated by repeated drying from three aliquots (0.5 ml) of methanol (Lindahl *et al.*, 1973). The products were redissolved in 200 μ l of water, reduced, and prepared for g.l.c. as described above for deamination at pH 3.5.

N.m.r.

Samples of VSG C-terminal glycopeptide were dissolved in 99.8% ²H₂O and freeze-dried several times before being dissolved in 100% ²H₂O (0.4 ml). ¹H-n.m.r. spectra were measured at 360 MHz on a Bruker WM 360 instrument at 25 °C. Free induction decays covering a spectral width of 3000 Hz were acquired into 32 K data points by using 45° pulses and a repetition rate of 5.47 s. A total of 1500 transients were acquired, and exponential multiplication equivalent to a 0.2 Hz line-broadening was applied before Fourier transformation. Acetone at 2.225 p.p.m. was used as a secondary chemical-shift (δ) reference.

RESULTS AND DISCUSSION

Deamination with HNO₂

HNO₂ cleaves the anomeric glycosidic glucosamine bond by causing deamination of the glucosamine followed by ring contraction, leaving 2,5-anhydromannose as the reducing terminal monosaccharide residue (reviewed by Horton & Philips, 1973). The reaction is dependent upon the substitution of the amino group on the glucosamine and the pH of the reaction mixture (Shively & Conrad, 1976). The yield of 2,5-anhydromannose is also variable, as it is not the only product formed. The pH optimum for the deamination of glucosamine with a free amino group is about 4.0, whereas that for *N*-sulphated glucosamine is less than 2.5. Hence, at pH 3.5, only compounds containing glucosamine with a free amino group will react completely, giving 2,5-anhydromannose, although *N*-sulphated glucosamine will react to some extent. However, at pH 1.5, only *N*-sulphated glucosamine will react. Therefore a distinction can be made between glucosamine (with a free amino group), *N*-sulphated glucosamine and *N*-acetylated glucosamine, which does not react at either pH value. The

Table 1. Generation of 2,5-anhydromannitol by HNO₂ deamination and reduction

Untreated and deaminated samples were prepared as described in the text and examined by g.l.c. for the presence/absence of 2,5-anhydromannitol. Abbreviation used: n.d., not determined.

pH of deamination...	Presence (+) or absence (–) of 2,5-anhydromannitol			
	1.5		3.5	
	Untreated	Deaminated	Untreated	Deaminated
Heparin	–	+	–	n.d.
GlcN	–	–	–	+
GlcNAc	–	–	–	–
Neutral monosaccharides	–	–	–	–
C-Terminal glycopeptide of VSG 121	–	–	–	+

Fig. 1. G.l.c. on untreated and deaminated C-terminal glycopeptide of VSG 121

Samples were deaminated with HNO_2 and examined by g.l.c. as described in the text. (a) Untreated; (b) deaminated at pH 1.5; (c) deaminated at pH 3.5. Arrowed positions of peaks: 1, 2,5-anhydromannitol; 2, mannose 1 + galactose 1; 3, mannose 2 + galactose 2; 4, galactose 3; 5, mannitol (internal standard). The peaks are pertrimethylsilyl derivatives of alditols or methyl glycosides. From Kamerling *et al.* (1975) the derivatives of the glycosides can be identified as: mannose 1 = $\text{Me}\alpha\text{Manp}$; mannose 2 = $\text{Me}\beta\text{Manp}$; galactose 1 = $\text{Me}\beta\text{Manp}$; galactose 2 = $\text{Me}\alpha\text{Galp} + \text{Me}\alpha\text{Galf}$; galactose 3 = $\text{Me}\beta\text{Galp}$.

generation of 2,5-anhydromannose as determined by g.l.c. after its reduction, methanolysis and trimethylsilylation from the HNO_2 deamination at pH 1.5 and pH 3.5 of glucosamine, *N*-acetylglucosamine and heparin are shown in Table 1, together with the results for the C-terminal glycopeptide of VSG 121. Heparin (Paines and Byrne, Greenford, Middx., U.K.) was used as the standard for *N*-sulphated glucosamine, and neutral monosaccharides were included as a control to establish that any 2,5-anhydromannitol observed in a deaminated

glycopeptide sample had not arisen from the neutral monosaccharides of the glycopeptide.

As expected, *N*-acetylglucosamine did not yield 2,5-anhydromannitol at either pH value, whereas glucosamine reacted only at pH 3.5, and heparin reacted at pH 1.5. The C-terminal glycopeptide of VSG 121 yielded 2,5-anhydromannitol at pH 3.5 and not at pH 1.5 (Fig. 1). The quantities of mannose and galactose, identified by their R_F value relative to mannitol ($R_{\text{Man-ol}}$) were unchanged by deamination at either pH value (results not shown), but an extra peak in the chromatogram of pH 3.5-deaminated C-terminal glycopeptide was noted and identified as 2,5-anhydromannitol by its $R_{\text{Man-ol}}$ value (2,5-anhydromannitol was defined as the product of pH 3.5-deaminated glucosamine).

Definitive proof of the presence of 2,5-anhydromannitol in pH 3.5-deaminated C-terminal glycopeptide was obtained by performing a series of co-chromatography experiments (results not shown), which showed that the 2,5-anhydromannitol peak, although small, was genuine. The results demonstrate that the glucosamine in the C-terminal glycopeptide of VSG 121 is not *N*-acetylated nor substituted by sulphate. Although we did not have the appropriate model compound, we would presume that any other neutral substituent, such as an *N*-glycosyl group, would behave in a similar manner to an *N*-acetyl group.

^1H n.m.r.

A 360 MHz ^1H -n.m.r. spectrum of the C-terminal glycopeptide is shown in Fig. 2. Signals characteristic of anomeric protons were observed in the chemical-shift range 5.0–5.6 p.p.m. and exhibited coupling constants of 3.5 or 1.5 Hz. The former was typical of α -galactose or α -glucose (or therefore of α -glucosamine), the latter was typical of mannose. At about 3.0 p.p.m., signals characteristic of the non-equivalent methylene group of aspartic acid were observed. Irradiation of the methylene protons caused a multiplet signal to collapse from a doublet of doublets to a singlet at about 4.3 p.p.m., characteristic of the α -CH of aspartate.

N-Acetyl protons would be expected to give sharp singlet resonances at 2.0–2.1 p.p.m., but only a broad low-intensity signal was observed in this position. Integrating this broad signal against a one-proton multiplet signal of aspartate methylene showed that they were about equal in area. An estimate of the possible presence of *N*-acetyl groups was made by a comparison of the maximum height of the broad signal (at 2.0–2.1 p.p.m.) with the total height of the one-proton multiplet signal of aspartate methylene. This showed that *N*-acetyl groups could not be present at any level greater than 0.05 mol/mol of aspartate, assuming that an *N*-acetyl signal would be of similar lineshape to those of the aspartate methylene protons. Signals from anomeric protons were of various intensities. Comparison of the integrals of the six resolved glucose/galactose signals against the one-proton integral of aspartate showed that the most intense signals correspond to 1 molar-equivalent concentration and the weakest to at least 0.2 molar equivalents. Although the anomeric signal of glucosamine has not been identified, the signal from an *N*-acetyl group at 0.2 molar-equivalent concentration or greater would have been detected above the broad signal at about 2.0 p.p.m.

Fig. 2. ^1H -n.m.r. spectrum of the C-terminal glycopeptide of VSG 121

The C-terminal glycopeptide of VSG 121 was prepared and the ^1H -n.m.r. spectrum acquired as described in the text. The chemical-shift scale in this spectrum has been referenced to a subsequent spectrum of the same sample with acetone at low Y-scaling showing only an $^1\text{H}_2\text{O}$ solvent signal at 4.8 p.p.m. The upper trace is the spectrum at increased Y-scaling showing signals from the glycopeptide. The upper integral trace compares the relative areas of one anomeric proton signal at 5.6 p.p.m. with the signals from an unidentified proton and the aspartate non-equivalent methylene protons at about 3.0 p.p.m. and with the broad signal at about 2.0 p.p.m. This shows the anomeric proton to be at about a molar equivalent concentration with respect to aspartate but that any *N*-acetyl is at a much lower concentration. The absence of a sharp singlet at about 2.0 p.p.m. shows that there is no significant amount of *N*-acetyl in the glycopeptide.

General discussion

HNO_2 treatment of glycosidically linked glucosamine results in nitrosation of the amino group, followed by deamination and ring contraction. The reactions of *N*-sulphated and free amino glucosamine are believed to proceed via the same reaction intermediate giving the same products. The amino group must be unacetylated and unprotonated (Shively & Conrad, 1976).

HNO_2 treatment was used in the structural analysis of the C-terminal glycopeptide of VSG 121 because of its selective reaction. The amino group of the glucosamine in this glycopeptide has been shown to be not *N*-acetylated and most probably to be free. The low yield of 2,5-anhydromannose was not unexpected, since this product is not the only product of glucosamine deamination by HNO_2 . The reaction is also dependent on the configuration of the anomeric glucosamine linkage, since α -linked glucosamine is released more slowly than its β -anomer (Foster *et al.*, 1953). The absence of an *N*-acetyl group is unequivocally confirmed by the ^1H -n.m.r. spectrum, which indicates the absence of *N*-acetyl groups in the C-terminal glycopeptide.

It is noteworthy that the immunological cross-reaction of the VSG can be abolished by HNO_2 treatment (Holder, 1983b). It is now evident that this is due to cleavage of the molecule at the glucosamine residue. The C-terminal oligosaccharide of the VSGs is obviously an unusual moiety, since it has already been shown to be linked to the protein via ethanolamine and to have a linkage to myristic (tetradecanoic) acid in the membrane form of the VSG. The presence of unsubstituted glucosamine is another novel feature of the structure which suggests that the biosynthesis of this part of the glycoprotein may offer an attractive target for directed chemotherapy of *Trypanosoma brucei* infections.

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