O-Glycosidically linked *N*-acetylglucosamine-bound oligosaccharides from glycoproteins of *Trypanosoma cruzi*

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In this report we describe studies on the structures of the O-linked carbohydrate units in cell-surface glycoproteins of epimastigote forms of the G-strain of *Trypanosoma cruzi*. Mild alkaline reductive degradation of the 38/43 kDa glycoproteins resulted in β-elimination of glycosylated threonine and/or serine residues, and the liberation of *N*-acetylglucosaminitol, galactobiosyl-, galactotriosyl-, galactotetraosyl- and galactopentaosyl-*N*-acetylglucosaminitol. The structures of these oligosaccharide alditols were established by n.m.r. spectroscopy and methylation analysis as: Gal$f\beta$1-4(Gal$p\beta$1-6)GlcNAc-ol; Gal$p\beta$1-3Gal$p\beta$1-6(Gal$f\beta$1-4)GlcNAc-ol; [(Gal$p\beta$1-3)(Gal$p\beta$1-2)Gal$p\beta$1-6](Gal$f\beta$1-4)GlcNAc-ol; [(Gal$p\beta$1-3)(Gal$p\beta$1-2)Gal$p\beta$1-6](Gal$p\beta$1-2Gal$f\beta$1-4)GlcNAc-ol.

INTRODUCTION

Trypanosoma cruzi is the causative agent of Chagas' disease, which affects 6 million individuals in Latin America (Garcia-Zapata et al., 1991). This flagellated protozoan has a complex life cycle involving a total of four developmental stages alternating between insect (triatomine bugs) and mammalian hosts. In the triatomine bug non-infective epimastigotes multiply by binary fission and differentiate into infective non-dividing metacyclic trypomastigotes which, after invasion of the mammal, are transformed into replicative intracellular amastigotes, which differentiate into infective non-dividing trypomastigotes which can infect new host cells or be ingested by a triatomine bug (Brener, 1973).

Characterization of the cell-surface glycoconjugates of the various developmental stages of *T. cruzi* is important because of their role in the interaction of the parasite and the cells of its hosts. In a previous report we described cell-surface glycoconjugates from epimastigotes of the Y-strain of *T. cruzi* that migrated on SDS/PAGE as a broad band centred at 43 kDa. These components were sialylated only when the parasites were grown or incubated with sialic acid-containing molecules (Previato et al., 1985). We therefore suggested that sialic acid was incorporated into *T. cruzi* macromolecules via a novel metabolic pathway involving a trans-sialidase reaction, rather than the more usual CMP-sialic acid-dependent sialyltransferase.

Piras et al. (1987) observed that sialylation of *T. cruzi* components was required for parasite entry into mammalian cells, and Schenkman et al. (1991) subsequently showed that attachment to and invasion of mammalian cells by trypomastigotes were regulated by addition of sialic acid to glycoproteins, collectively designated Stage-specific antigens 3 (Ssp3).

It is known that the *T. cruzi* trans-sialidase catalyses the transfer of sialic acid residues linked α-2,3- (but not α-2,6- or α-2,8-) from the donor substrate to the 3-*O* position of non-reducing end units of β-D-galactopyranose (Vandekerckhove et al., 1993; Ferrero-Garcia et al., 1993). However, the structures of the saccharide moieties of *T. cruzi* glycoconjugates acting as sialic acid acceptors have not previously been determined. We now report the sequences of carbohydrate chains isolated from 38/43 kDa molecules purified from *T. cruzi* grown in the absence of sialic acid donors. Surprisingly, this study also demonstrates that *T. cruzi* epimastigote forms express cell-surface glycoproteins in which unsubstituted or substituted *N*-acetylglucosaminyl units are O-glycosidically attached to the hydroxyls of threonine and/or serine.

EXPERIMENTAL

Growth of parasites

A 20 ml portion of a fresh culture of *T. cruzi* G-strain epimastigotes was transferred to flasks containing 200 ml of brain heart infusion (BHI) supplemented with 10 mg/l haemin (BHI/haemin medium) and 5% fetal calf serum (FCS). These were incubated at 26 °C with shaking (80 rev./min) for 5 days. This 200 ml culture was used to inoculate 3-litre flasks containing 1 litre of BHI/haemin medium without FCS, other conditions being as described above. The cells were harvested by centrifugation, washed three times with 0.9% NaCl and frozen at −20 °C.

Isolation and purification of 38/43 kDa glycoconjugates

The 38/43 kDa glycoconjugates were isolated as previously described (Previato et al., 1985). Frozen cells were thawed and extracted three times with cold water. The resulting cell debris was extracted with aq. 45% phenol at 75 °C. The aqueous layer was dialysed, freeze-dried, dissolved in water and applied to a

Abbreviations used: BHI, brain heart infusion; d.q.f.-c.o.s.y., double-quantum-filtered correlation spectroscopy; f.a.b., fast-atom bombardment; FCS, fetal calf serum; FT, Fourier transformation; h.m.q.c., heteronuclear multiple quantum coherence; GalNAc-ol, *N*-acetylgalactosaminitol; GlcNAc-ol, *N*-acetylglucosaminitol; Neu^{7}Ac, 5-acetamido-3,5-dideoxy-L-*arabino*-2-heptulosonic acid; $[M+H]^+$, protonated molecule; n.O.e., nuclear Overhauser enhancement; r.o.e.s.y., rotating frame nuclear Overhauser enhancement spectroscopy; t.o.c.s.y., total correlation spectroscopy.

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column (2 cm × 100 cm) of Bio-Gel P-60. The excluded material was lyophilized, extracted with chloroform/methanol/water (10:10:3, by vol.), and the 38/43 kDa glycoconjugates were recovered by centrifugation, dissolved in water and lyophilized. The yield was about 100 mg/10^{12} cells.

Preparation of [7-^{3}H]Neu^7-Ac-labelled oligosaccharide alditols from fetuin

Fetuin labelled with ^{3}H at C-7 of sialic acid was prepared as described previously (Previato et al., 1985), and the labelled oligosaccharide chains were released by treatment with 1 M $NaBH_4$ in 1 M NaOH for 6 h at 100 °C. The pH was adjusted to 6.0 with 50% acetic acid and the solution was passed through Dowex 50W X8 (25–50 mesh; H^+ form). Boric acid was removed by repeated addition of methanol and evaporation to dryness. The residue was dissolved in water and applied to a Bio-Gel P-2 (extra fine) column (1 cm × 120 cm). Labelled oligosaccharides in the excluded volume were re-N-acetylated (Reading et al., 1978) and again purified on a Bio-Gel P-2 column, being then recovered in the void volume.

Biosynthetic labelling of 38/43 kDa glycoconjugates with [7-^{3}H]Neu^{7}Ac-labelled oligosaccharide alditols derived from fetuin

T. cruzi epimastigote forms grown in BHI/haemin medium were washed twice with phosphate/saline/glucose buffer, pH 7.0, containing 0.01 M PBS/1% glucose. The cells were suspended in the same buffer containing fetuin-derived [7-^{3}H]Neu^{7}Ac-labelled oligosaccharides (9×10^5 c.p.m.) at a concentration of 5×10^7 cells/ml and were incubated for 2 h at 26 °C. A portion of the labelled cells was exhaustively washed with phosphate/saline/glucose buffer and then directly solubilized in electrophoresis sample buffer consisting of 0.5 M Tris/HCl, pH 6.8, 2% SDS, 5% glycerol and 3% 2-mercaptoethanol before analysis by SDS/PAGE. Autoradiography of the dried gels was by fluorography at −80 °C (Gahmberg, 1978).

Alkaline degradation and peptide–*N*-glycosidase treatment of [7-^{3}H]Neu^{7}Ac-labelled 38/43 kDa glycoproteins

The labelled 38/43 kDa glycoproteins were isolated and purified as described above, and submitted to reductive β-elimination in 100 mM NaOH solution containing 0.3 M $NaBH_4$. After 48 h at 37 °C the reaction was terminated by addition of 1 M acetic acid and freeze-dried. The residue was applied to a column (1.3 cm × 150 cm) of Bio-Gel P-6 (200–400 mesh) equilibrated with 0.1 M pyridine acetate, pH 5.0, and elution of [7-^{3}H]Neu^{7}Ac oligosaccharide alditols was monitored by liquid-scintillation counting.

Labelled 38/43 kDa glycoproteins were treated with *N*-glycosidase F from *Flavobacterium meningosepticum* in 50 mM Tris/HCl buffer, pH 8.6, at 37 °C for 18 h as described by Tarentino et al. (1985).

Alkaline reductive degradation of 38/43 kDa glycoconjugates

The 38/43 kDa glycoconjugates (70 mg) were subjected to β-elimination in 100 mM NaOH solution containing 0.3 M $NaBH_4$ (7 ml) at 37 °C for 18 h. The reaction was terminated by neutralization with 1 M acetic acid, and the resulting solution was passed through Dowex 50W X8 (25–50 mesh; H^+ form) and lyophilized. Boric acid was removed by repeated additions of methanol and evaporation to dryness. The residue was taken up in 0.05 M acetic acid and fractionated on a Bio-Gel P-4 (extra fine) column (1 cm × 120 cm), which was eluted with 0.05 M acetic acid. Fractions of 1.5 ml were collected and assayed by spotting 5 μl portions on a t.l.c. plate and staining with orcinol/H_2SO_4 reagent (Humbel and Collaert, 1975) and H_2SO_4 spray (Wing and BeMiller, 1972).

Carbohydrate analysis

For analysis of neutral and amino sugars, 38/43 kDa glycoconjugates and purified β-eliminated oligosaccharide alditols were subjected to methanolysis (0.5 M HCl in methanol containing xylitol as internal standard), for 18 h at 80 °C, followed by neutralization with silver carbonate and re-N-acetylation with acetic anhydride. The dried residue was trimethylsilylated by addition of bis(trimethylsilyl)trifluoroacetamide/pyridine (1:1, v/v) (Sweeley et al., 1963). The products were analysed by g.l.c. on a DB-1 fused silica column (30 m × 0.25 mm internal diameter) using hydrogen as the carrier gas at 0.7×10^5 Pa. The column temperature was programmed from 120° to 240 °C at 2 °C/min.

Methylation analysis

Permethylation of β-eliminated oligosaccharide alditols was performed by the method of Ciucanu and Kerek (1984) as modified by Previato et al. (1990). The partially methylated and acetylated methyl glycosides were analysed by g.l.c. on a DB-1 fused silica column (30 m × 0.25 mm internal diameter) using hydrogen as the carrier gas at 0.7×10^5 Pa. The column oven temperature was increased from 120° to 240 °C at 2 °C/min. The separated components were identified by their relative retention times and by g.l.c.–m.s. (Hase and Rietschel, 1976; Fournet et al., 1981) and were quantified by their peak areas.

N.m.r.

Samples for n.m.r. spectroscopy were deuterium exchanged by repeated lyophilization from deuterium oxide, and dissolved in 0.7 ml of ^2H_2O before analysis. N.m.r. spectra were acquired on a Varian Unity 500 n.m.r. Spectrometer (Varian Associates, Palo Alto, CA, U.S.A.) equipped with a 5 mm triple-resonance probe (for proton-detected experiments) or a 5 mm broadband probe (for ^{13}C-detected experiments) at an indicated probe temperature of 30 °C. Chemical shifts (δ) are expressed in p.p.m. Proton spectra were referenced against internal acetate anion at 1.908 p.p.m. and carbon spectra against internal acetone at 31.5 p.p.m. When water presaturation was employed, this was achieved using a transmitter pulse. Phase-sensitive two-dimensional spectra were obtained using the method of States et al. (1982). Typical experimental conditions were as follows. 1D-proton spectra: 16K datapoints were collected over a sweep width of 5000 Hz and the data zero-filled to 32K and weighted with a shifted sine-bell function before transformation. 1D-carbon spectra: typically, 16K datapoints were collected over a 25 kHz spectral width, using broadband proton decoupling; the pulse angle was 70° and a 1 s relaxation delay was left between scans; the data were zero-filled to 32K before Fourier transformation (FT). Double-quantum filtered correlation spectroscopy (d.q.f.-c.o.s.y.) (Piantini et al., 1982; Rance et al., 1983): 16 transients of 4032 datapoints were averaged for each free induction decay, and 512 × 2 increments were collected in f1; spectral widths of 4000 Hz were collected in both frequency

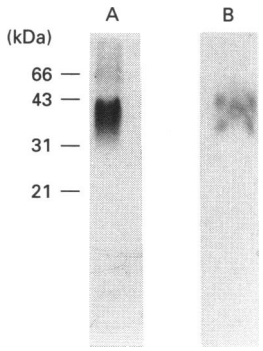

Figure 1 SDS/PAGE of 38/43 kDa glycoprotein preparations

Lane A, periodate/Schiff staining of purified 38/43 kDa glycoproteins of *T. cruzi* G-strain; lane B, autofluorography of biosynthetic labelling of live epimastigotes with [7-^{3}H]Neu^{7}Ac oligosaccharide alditols derived from labelled fetuin. Positions of molecular-mass (*M*) markers [BSA (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (31 kDa), soya-bean trypsin inhibitor (21 kDa)] are shown.

Table 1 Amino acid composition of 38/43 kDa glycoproteins of *T. cruzi* epimastigote forms

Amino acid	Composition (residues/100 residues)
Asx	15.3
Glx	4.9
Ser	14.5
Gly	6.9
His	0.0
Arg	0.0
Thr	35.5
Ala	9.0
Pro	9.2
Tyr	0.0
Val	1.8
Met	0.0
Ile	0.3
Leu	0.4
Phe	0.1
Lys	2.1

domains; shifted sine-bell weighting functions were applied in both domains; the f1 data were zero-filled to 2048 points before the second FT; the residual HO^{2}H signal was attenuated by pre-irradiation for 0.5 s. Total correlation spectroscopy (t.o.c.s.y.) (Braunschweiler and Ernst, 1983): 16 transients of 4032 datapoints were averaged for each free induction decay, and 512 × 2 increments were collected in f1; spectral widths of 3000 Hz were collected in both frequency domains; the 7.1 kHz spinlock field was applied for 80 ms; shifted sine-bell weighted functions were applied in both domains; the f1 data were zero-filled to 2048 points before the second FT; the residual HO^{2}H signal was attenuated by pre-irradiation for 0.5 s. Rotation frame nuclear Overhauser enhancement spectroscopy (r.o.e.s.y.) (Bothner-By et al., 1984; Kessler et al., 1987): 32 transients of 4032 datapoints were averaged for each free induction decay, and 512 × 2 increments were collected in f1; spectral widths of 3000 Hz were collected in both frequency domains; the 2.8 kHz spinlock field

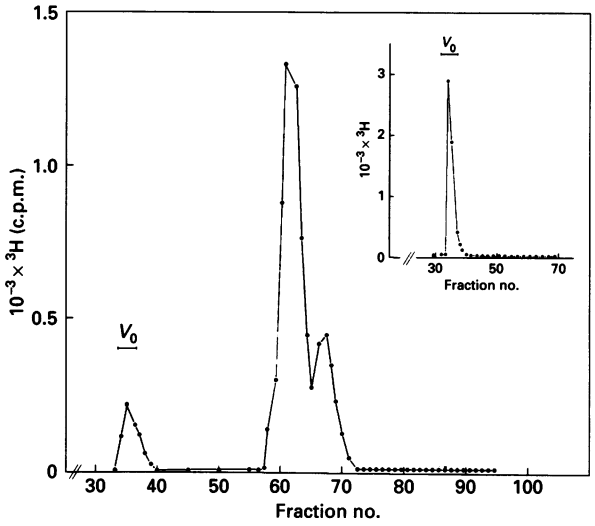

Figure 2 Gel-filtration chromatography of native and β-eliminated [7-^{3}H]Neu^{7}Ac-labelled 38/43 kDa glycoproteins of *T. cruzi* G-strain

Bio-Gel P-6 gel-filtration chromatograms of labelled 38/43 kDa glycoproteins (inset) and the oligosaccharide alditols released by reductive β-elimination from labelled 38/43 kDa glycoproteins of *T. cruzi* are shown. Fraction size, 2.0 ml. Experimental conditions were as described in the Experimental section.

was applied for 150 ms; shifted sine-bell weighted functions were applied in both domains; The f1 data were zero-filled to 2048 points before the second FT; the residual HO^{2}H signal was attenuated by pre-irradiation for 0.5 s. Heteronuclear multiple quantum coherence (h.m.q.c.) (Summers et al., 1986; Lerner and Bax, 1987): 16 transients of 1932 datapoints were averaged, and, typically, 500 × 2 increments in f1 were collected; the f2 data were zero-filled to 4K datapoints and the f1 data to 2K datapoints before transformation; data were weighted with a shifted sine-bell function before transformation; typical spectral accumulation time was 16 h.

Fast atom bombardment (f.a.b.)-m.s.

Positive-ion f.a.b. mass spectra were acquired using a Kratos MS80 RFA spectrometer, fitted with an Ion Tech saddle field atom gun using xenon as the bombarding gas. A 1:1 mixture of glycerol and dithiothreitol/dithioerythritol (5:1, v/v) was used as liquid matrix for both native and peracetylated samples. Collision-induced dissociation was achieved by admitting helium into a gas cell in the field-free region between the ion source and the electrostatic analyser. The decomposition products were analysed by scanning the electric (E) and magnetic (B) fields so as to maintain a constant ratio of B/E.

Samples were peracetylated by treatment with a 1:1 mixture of trifluoroacetic anhydride and acetic acid for 10 min at room temperature. The reagents were removed in a vacuum centrifuge, and the acetylated oligosaccharides were dissolved in 1 ml of chloroform and washed three times with an equal volume of distilled water. The chloroform was evaporated to dryness and the residue dissolved in methanol before f.a.b.-m.s. analysis.

Other methods

Total neutral sugars were analysed by the phenol/H_2SO_4 procedure (Dubois et al., 1956), protein was determined by the

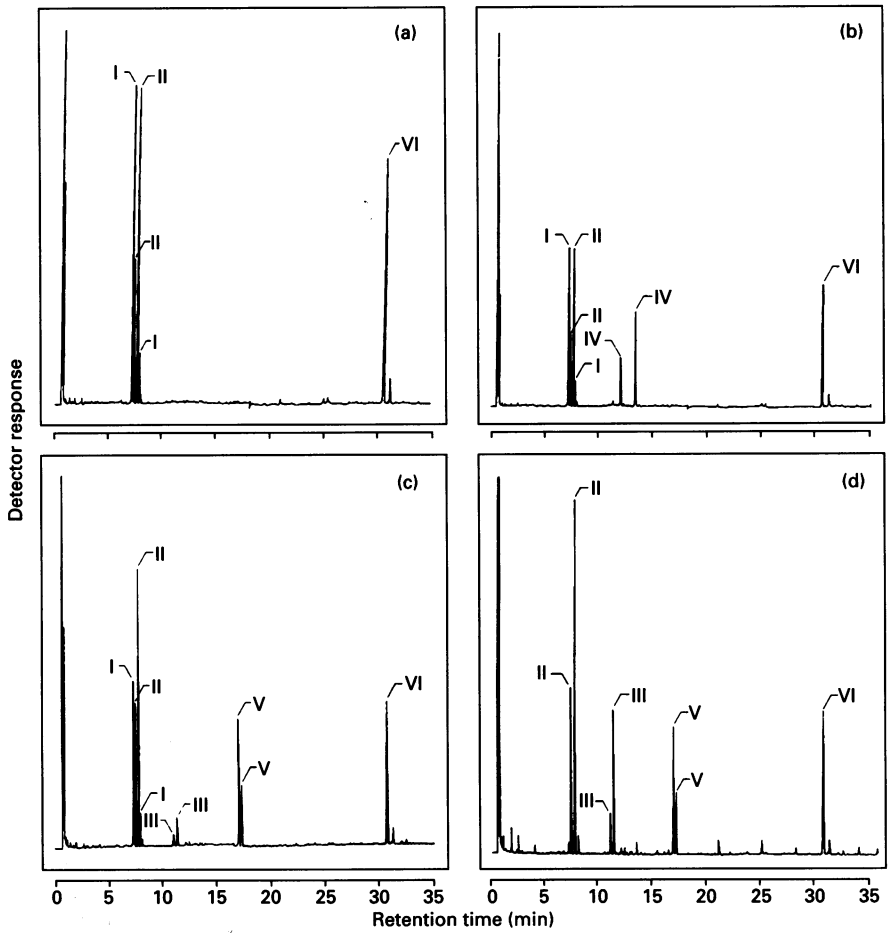

Figure 3 Methylation analysis of the oligosaccharide alditols of *T. cruzi* 38/43 kDa glycoproteins

G.l.c. of O-acetylated partially O-methylated methyl glucosides of (**a**) disaccharide alditol, (**b**) trisaccharide alditol, (**c**) tetrasaccharide alditol and (**d**) pentasaccharide alditol. Derivatives were designated as follows: I, methyl 2,3,5,6-tetra-*O*-methylgalactofuranoside; II, methyl 2,3,4,6-tetra-*O*-methylgalactopyranoside; III, methyl 3,5,6-tri-*O*-methyl-2-mono-*O*-acetylgalactofuranoside; IV, methyl 2,4,6-tri-*O*-methyl-3-mono-*O*-acetylgalactopyranoside; V, methyl 4,6-di-*O*-methyl-2,3-di-*O*-acetylgalactopyranoside; VI, 4,6-di-*O*-acetyl-1,3,5-tri-*O*-methyl-2-deoxy-2-(*N*-methylacetamido)glucitol.

method of Lowry et al. (1951), total phosphorus by the method of Ames (1966), hexosamine by modification of the Elson–Morgan reaction (Belcher et al., 1954), and sialic acid by the thiobarbituric acid method (Warren, 1959). Amino acids were determined after strong acid hydrolysis (6 M HCl, 110 °C, 16 h) using a Waters Pico-Tag System (Schneider et al., 1990).

SDS/PAGE was performed as described by Laemmli (1970) on a 15% acrylamide gel. Glycoconjugates were detected with the periodate/Schiff reagent (Fairbanks et al., 1971).

Partial acid hydrolysis of oligosaccharide alditols was carried out with 0.04 M trifluoroacetic acid for 1 h at 100 °C. The products were separated by gel-filtration chromatography on a Bio-Gel P-2 (extra fine) column (0.5 cm × 100 cm).

RESULTS

Isolation and chemical composition of the 38/43 kDa glycoproteins of *T. cruzi*

T. cruzi was cultured in the absence of FCS, and the harvested cells were extracted with hot phenol/water. A chloroform/methanol/water (10:10:3, by vol.)-insoluble carbohydrate-containing fraction was recovered from the aqueous phase which, when analysed by SDS/PAGE, gave a broad band of 38/43 kDa which was stained by periodate/Schiff reagent, but not with Coomassie Brilliant Blue (Figure 1). Compositional analysis revealed neutral sugars (57.5%), protein (4.5%), phosphorus (1%), hexosamine (16%) and traces of sialic acid. Carbohydrate analysis revealed Gal, Man, GlcNAc and GalNAc in a molar ratio of 4.8:1:1.6:0.15. The low amount of protein detected by the Lowry method could be due to the high content of threonine and serine (50% of total amino acids) in the 38/43 kDa glycoproteins (Table 1).

[7-^{3}H]Neu^{7}Ac incorporation into 38/43 kDa glycoproteins

T. cruzi epimastigote forms grown in the absence of FCS were incubated for 2 h in phosphate/saline/glucose buffer containing [7-^{3}H]Neu^{7}Ac-labelled oligosaccharide alditols derived from fetuin. A portion of the labelled cells was removed, washed, solubilized in electrophoresis buffer and analysed by SDS/PAGE. As shown in Figure 1, radioactivity was exclusively incorporated into the 38/43 kDa band, demonstrating that in the epimastigote stage of *T. cruzi* these cell-surface glycoconjugates are the main acceptors for sialic acid incorporation by the trans-sialidase reaction.

Evidence that [7-^{3}H]Neu^{7}Ac was incorporated into O-linked oligosaccharides was obtained after reductive β-elimination of labelled 38/43 kDa glycoproteins. Examination of the products

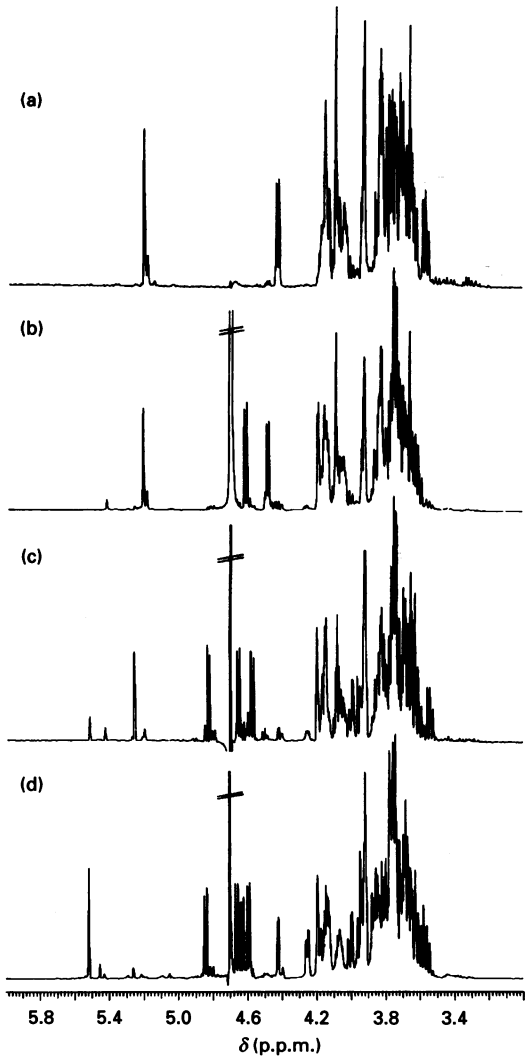

Figure 4 ^{1}H-n.m.r. spectroscopy of the oligosaccharide alditols of *T. cruzi* 38/43 kDa glycoproteins

Partial 500 MHz ^{1}H-n.m.r. spectra of (**a**) disaccharide alditol (the resonance from residual HO^2H is suppressed by transmitter presaturation), (**b**) trisaccharide alditol, (**c**) tetrasaccharide alditol and (**d**) pentasaccharide alditol. Presaturation was not used for these oligosaccharide alditols to present partial saturation of the Gal*p* resonances. Experimental conditions were as described in the Experimental section.

of this reaction by gel-filtration chromatography on a Bio-Gel P-6 column (Figure 2) showed inclusion of radioactive fractions, corresponding to [7-^{3}H]Neu^{7}Ac-labelled oligosaccharide alditols. Also, the treatment of labelled 38/43 kDa glycoproteins with *N*-glycosidase F did not release radiolabelled oligosaccharides included in the Bio-Gel P-6 column.

Fractionation of β-elimination reduced oligosaccharides

Oligosaccharide alditols were released from 38/43 kDa glycoproteins by β-elimination and separated by gel-filtration chromatography on a Bio-Gel P-4 column; five included fractions were obtained (designated I–V), which were eluted at volumes corresponding to reduced di-, tetra-, penta-, hexa- and heptasaccharides. A series of reduced (1→4)-linked α-D-glucopyranosyl oligosaccharide standards was used to calibrate the column. The molar ratios of fractions I to V were 1:1.5:0.5:1.5:0.5 respectively.

Characterization of purified oligosaccharide alditols

The β-eliminated reduced oligosaccharides were methanolysed and their sugar compositions determined by g.l.c. after trimethylsilylation. Fractions II, III, IV and V consisted of Gal, *N*-acetylglucosaminitol (GlcNAc-ol) and *N*-acetylgalactosaminitol (GalNAc-ol) in the molar ratio of 2:1:0.08, 3:1:0.1, 4.2:1:0.09 and 5:1:0.07 respectively. These compositions were confirmed by f.a.b.-m.s., as protonated molecules, $(M+H)^+$, were observed at *m/z* 548, 710, 872 and 1034 corresponding to 1 mol of *N*-acetylhexosaminitol, together with two (disaccharide alditol), three (trisaccharide alditol), four (tetrasaccharide alditol) and five (pentasaccharide alditol) hexose residues in fractions II, III, IV and V respectively. Fraction I was shown by f.a.b.-m.s. [$(M+H)^+ = m/z$ 224] and g.l.c.–m.s. of its trimethylsilyl ether to contain only GlcNAc-ol. In addition to these molecular ions, the spectra of all the oligosaccharides contained abundant glycosidic cleavage ions. However, it proved impossible to assign unique structures on the basis of the conventional f.a.b. spectra of the native compounds, because the spectra were complicated by the presence of extensive sodium cationization, and because of the prevalence of multiple cleavage reactions in which the consecutive cleavage of more than one glycosidic bond results in 'second-generation' fragments which are indistinguishable from the products of 'first-generation' cleavages of a single glycosidic bond (Dell, 1987). For example, the positive-ion-mode f.a.b. spectrum of the disaccharide alditol contained two major fragment ions at *m/z* 386 and 224, which are equally compatible with the linear compound Hex-Hex-HexNAc-ol or with a branched structure in which the hexosaminitol is substituted with two hexose residues. Similarly, in the spectrum of the pentasaccharide alditol, Y-type reducing-terminal-containing ions [using the nomenclature introduced by Domon and Costello (1988)] were observed at *m/z* 872, 710, 548 and 224, which could suggest a linear arrangement of hexose residues rather than the branched structure subsequently deduced from the n.m.r. and methylation evidence. Some of these ambiguities were resolved by helium collisional activation of the protonated molecules of the tri-, tetra- and penta-saccharide alditols. In the case of the pentasaccharide alditol for example, the daughter ion spectrum of *m/z* 1034 (the protonated molecule) contained intense signals at *m/z* 872, 710 and 548 attributable to single glycosidic cleavages, whereas the ions at *m/z* 386 and 224 were very weak, suggesting that these latter are second-generation fragments. This result would not have been expected for a linear compound. The oligosaccharide alditols were also analysed by f.a.b.-m.s. after peracetylation. These derivatives are useful because the products of consecutive cleavages are easily recognized as they appear at lower mass than the corresponding first-generation fragment.

Interpretation of the spectra is further simplified because peracetylation sugars tend to fragment via pathways producing predominantly non-reducing-terminal-containing ions [of type B in the Domon and Costello (1988) nomenclature; rather confusingly some workers refer to these fragments as A-type oxonium ions (Dell, 1987)]. F.a.b.-m.s. of the peracetylated pentasaccharide alditol revealed, as expected, a protonated molecule at *m/z* 1874. Intense signals were observed at *m/z* 331, 619 and 907, which were assigned as B-type non-reducing ions. The absence of any further fragments of this type at *m/z* 1195 and 1483 strongly suggests that the compound is branched, with the

Table 2 ^{1}H- and ^{13}C-n.m.r. chemical shifts (p.p.m.) of the oligosaccharide alditols of *T. cruzi* 38/43 kDa glycoproteins

Proton chemical shifts are referenced to acetate anion at 1.908 p.p.m.

Oligosaccharide alditol	H-1	H-1′	H-2	H-3	H-4	H-5	H-6	H-6′	NAc	C-1	C-2	C-3	C-4	C-5	C-6	NAc
Disaccharide																
Gal *p*(1)	4.431	–	3.568	3.663	3.930	3.707*	3.664	–	–	104.41†	72.04†	73.88†	69.88†	76.39	62.24	–
Gal *f*	5.213	–	4.163	4.083	4.097*	3.816	3.729	3.634	–	109.25†	82.48†	77.38†	83.88	71.74	64.09	–
GlcNAc-ol	3.682	3.758	4.170	3.934	3.828	4.038	3.841	4.146	2.057	61.99*	53.90*†	69.49	78.96*†	70.90	71.56	23.40*
Trisaccharide																
Gal *p*(2)	4.617	–	3.609	3.669	3.926	–	–	–	–	105.52	72.31*	73.20*	69.84*	76.34*	62.22*	–
Gal *p*(1)	4.491	–	3.733	3.836	4.196	3.732	–	–	–	104.19*	71.26*	83.33*	69.71*	76.05*	62.22*	–
Gal *f*	5.217	–	4.154	4.083	4.096	3.830	3.732	3.641	–	109.29*	82.53*	77.43*	83.91*	71.78*	64.13*	–
GlcNAc-ol	3.681	3.758	4.171	3.934	3.830	4.048	3.849	4.148	2.058	62.02*	53.93*	69.53*	59.02*	70.96*	71.61*	23.40*
Tetrasaccharide																
Gal *p*(3)	4.833	–	3.550	3.646	3.926	3.689†	3.7	3.7	–	104.19†	72.39†	74.00‡	70.09‡	76.59‡	62.21‡	–
Gal *p*(2)	4.661	–	3.617	3.666	3.928	3.676†	3.7	3.7	–	105.2†	72.78†	74.02	69.92‡	72.36‡	62.21‡	–
Gal *p*(1)	4.580	–	3.958	4.008	4.205	3.734†	3.7	3.7	–	103.21†	72.20†	83.78†	69.97†	75.90†	62.13‡	–
Gal *f*	5.266	–	4.158	4.080	4.088	3.838	3.732	3.652	–	109.38†	82.71†	77.57†	83.29†	71.25†	64.11†	–
GlcNAc-ol	3.758	3.682	4.163	3.930	3.819†	4.048	3.843†	4.164	2.057	61.13†	53.20†	69.61†	79.05†	71.11†	72.06	23.40*
Pentasaccharide																
Gal *p*(4)	4.596	–	3.580	3.679	3.946	3.700	–	–	–	102.73	72.08	73.79‡	69.77‡	76.34‡	62.07	–
Gal *p*(3)	4.846	–	3.568	3.638	3.917	3.686	–	–	–	104.01	72.38	74.07‡	69.94‡	76.51‡	62.27	–
GAl *p*(2)	4.666	–	3.612	3.665	3.923	–	–	–	–	105.25	72.77	74.07‡	70.23‡	76.57‡	62.27	–
Gal *p*(1)	4.635	–	3.949	4.011	4.198	–	–	–	–	102.99	77.19	84.06	70.08	75.87	62.12	–
Gal *f*	5.522	–	4.427	4.257	4.141	3.873	3.754	3.676	–	103.32	88.80	76.96	84.51	71.93	64.13	–
GlcNAc-ol	–	–	4.140	3.926	3.844	4.065	–	4.166	2.058	62.12	53.81	69.63	78.73	70.99	71.76	23.40

* Assignment based on chemical-shift arguments (Bock and Pedersen, 1983).
† Assignment from h.m.q.c. spectrum.
‡ Assignments interchangeable.

longest branch containing three hexose residues. Consistent with this, weaker hexosaminitol-containing ions were observed at *m/z* 1544 and 1256, but not at lower mass, again ruling out a linear structure. Peracetylation and f.a.b.-m.s. of the other oligosaccharides also resulted in spectra which were consistent with the structures deduced from methylation analysis and n.m.r. spectroscopy.

Methylation analysis

Permethylated oligosaccharides were methanolysed, the products were acetylated and the resulting partially O-methylated *O*-acetyl methyl glycosides were characterized by g.l.c.–m.s. The methylation products of the disaccharide alditol (Figure 3a) showed the presence of methyl 2,3,5,6-tetra-*O*-methylgalactofuranoside, methyl 2,3,4,6-tetra-*O*-methylgalactopyranoside and 4,6-di-*O*-acetyl-1,3,5-tri-*O*-methyl-2-deoxy-(*N*-methylacetamido)glucitol, indicating that this oligosaccharide contained a terminal GlcNAc-ol residue substituted at positions 4 and 6 by Gal units. These derivatives were also obtained from the trisaccharide alditol, which additionally produced methyl 2,4,6-tri-*O*-methyl-3-mono-*O*-acetylgalactopyranoside (Figure 3b). To determine the positions of attachment of the galactofuranosyl and galactopyranosyl residues, the disaccharide alditol was subjected to partial acid hydrolysis, and the products were separated on a column of Bio-Gel P-2. Two carbohydrate fractions were recovered, one containing only Gal, and the other an equimolar mixture of Gal and GlcNAC-ol. Methylation analysis of this latter peak resulted in methyl 2,3,4,6-tetra-*O*-methylgalactopyranoside and 6-mono-*O*-acetyl-1,3,4,5-tetra-*O*-methyl-2-(*N*-methylacetamido)glucitol. These results revealed that the GlcNAc-ol unit was substituted on O-4 by a Gal*f* and on O-6 by Gal*p* units in the disaccharide alditol. This linkage pattern was confirmed for the trisaccharide and tetrasaccharide alditols by the same procedure of partial hydrolysis and methylation analysis. The methylation products from the tetrasaccharide alditol differed from those of the trisaccharide alditol by the presence of 2,3-di-O-substituted Gal*p* in place of the 3-O-substituted Gal*p* and an additional terminal Gal*p* residue. These results are consistent with a tetrasaccharide alditol, in which the Gal*p* adjacent to GlcNAc-ol is substituted in positions 2 and 3 by non-reducing Gal*p* units. Methylation analysis (Figure 3) showed that the pentasaccharide alditol differed from the tetrasaccharide alditol in that the galactofuranosyl residue linked to position 4 of GlcNAc-ol was substituted in position 2 by a non-reducing Gal*p* unit.

N.m.r. spectroscopy of oligosaccharide alditols

The n.m.r. data for the oligosaccharide alditols are in the agreement with the structures suggested by methylation analysis. ^{1}H-n.m.r. spectra of purified oligosaccharide alditols (Figure 4) contain one low-field anomeric resonance at 5.2 or 5.5 p.p.m. with a small coupling constant ($^{3}J_{1,2}$ about 2 Hz in di-, tri- and tetra-saccharide alditols and not resolved in pentasaccharide alditol) characteristic of a βGal*f* unit, and an increasing number of higher-field anomeric signals ranging from 4.85 to 4.4 p.p.m. ($^{3}J_{1,2}$ 8 Hz) typical of β-Gal*p* residues. Proton assignments were obtained from a combination of d.q.f.-c.o.s.y. and t.o.c.s.y. experiments (Table 2). Assignments of the spin systems were complicated by spectral overlap and some weak correlation peaks, and some proton assignments only became clear when the ^{13}C-n.m.r. spectra were assigned. For instance, in the disaccharide alditol, no cross-correlation peak was observed between β-Gal*p*

H-4 and H-5 because of the small coupling constant, but the β-Gal*p* H-5 chemical shift could be determined from the h.m.q.c. spectrum; the assignment of the β-Gal*p* C-5 was made on chemical-shift arguments. Similarly, the high-field methine resonance (53.9 p.p.m.) was assigned as C-2 of GlcNAc-ol on chemical-shift grounds (Bock and Pedersen, 1983), and the assignment of the GlcNAc-ol H-2 resonance was established unambiguously from the h.m.q.c. spectrum. Carbon assignments for disaccharide alditol were determined from an h.m.q.c. spectrum without carbon decoupling.

The proton and carbon assignments for both Gal residues in the disaccharide alditol were consistent with them being terminal, as determined by methylation analysis (Figure 3), and the β-anomeric configuration of the Gal*f* residue was confirmed from the carbon chemical shifts and the $^1J_{C,H}$ of the C-1 (175.5 Hz) (Bock and Pedersen, 1983). The linkage of these Gal residues to the GlcNAc-ol was confirmed by a long-range carbon–proton correlation experiment. The β-Gal*f* H-1 showed strong correlation with the β-Gal*f* C-4 and the GlcNAc-ol C-4, and weak correlations with β-Gal*f* C-2 and C-3. The β-Gal*f* C-1 showed a correlation with the GlcNAc-ol H-4 (3.828 p.p.m.). The β-Gal*p* C-1 showed correlations with the GlcNAc-ol H-6 and (weakly) H-6′, and the β-Gal*p* H-1 showed a strong correlation with the GlcNAc-ol C-6. The main characteristic of 2D proton spectra of trisaccharide alditol compared with those of disaccharide alditol is the presence of an additional β-Gal*p* spin system (H-1 at 4.617 p.p.m.) (Table 2) which is consistent with a non-reducing terminal residue. The low field position of the β-Gal*p* C-3 resonance (83.33 p.p.m. in the trisaccharide alditol) (Table 2 and Figure 5) indicates a -Gal*p*β1-3Gal*p*β1 substructure. Compared with the trisaccharide alditol, the 2D proton spectra of the tetrasaccharide alditol showed the presence of an additional terminal β-Gal*p*(3) spin system (H-1 at 4.833 p.p.m.) (Table 2) and changes in the chemical shifts of the internal β-Gal*p*(1) residue, consistent with the presence of a branch point, as indicated by the methylation analysis (Figure 3). The r.o.e.s.y. spectrum obtained with a 150 ms mixing time showed cross-peaks between the low-field β-Gal*p*(3) anomeric resonance and the H-2 of the branch point β-Gal*p*(1), and between the anomeric of the β-Gal*p*(2) and β-Gal*p*(1) H-3 and H-4. Strong t.o.c.s.y. artifacts were observed for

Table 3 Summary of inter- and intra-residue n.O.e.s observed for the oligosaccharide alditols (tetra- and penta-saccharide alditols)

Oligosaccharide alditol	Inter- and intra-residue n.O.e.s		
Tetrasaccharide			
β-Gal*f* H-1	H-5 @ 3.858†	GlcNAc-ol	H-4 @ 3.825
	H-6 @ 3.658†	GlcNAc-ol	H-3 @ 3.927
	H-6 @ 3.751†	GlcNAc-ol	H-5 @ 4.046
		β-Gal*p*(1)	H-1 @ 4.582
β-Gal*p*(3) H-1	H-3 @ 3.646	β-Gal*p*(1)	H-2 @ 3.954
	H-5 @ 3.696	β-Gal*p*(1)	H-3 @ 4.008
β-Gal*p*(2) H-1	H-2 @ {3.672}	β-Gal*p*(1)	H-3 @ 4.000
		β-Gal*p*(1)	H-4 @ 4.207
β-Gal*p*(1) H-1	H-2 @ {3.950}	GlcNAc-ol	H-6 @ 4.164
	H-3 @ {4.008}	GlcNAc-ol	H-6′ @ 3.850
	H-4 @ {4.204}		
	H-5 @ 3.750		
	H-6 @ 3.792		
β-Gal*p*(1) H-6	H-5 @ 3.742		
	H-6 @ 3.783		
Pentasaccharide‡			
β-Gal*f* H-1	H-2 @ 4.441	GlcNAc-ol	H-4 @ 3.850
		GlcNAc-ol	H-3 @ 3.926
		GlcNAc-ol	H-5 @ 4.067
		β-Gal*p*(1)	H-1 @ 4.365
β-Gal*p*(3) H-1	H-5 @ 3.700	β-Gal*p*(1)	H-2 @ 3.967
	H-3 @ 3.650	β-Gal*p*(1)	H-3 @ 4.015*
	H-2 @ 3.588		
β-Gal*p*(2) H-1	H-3 @ 3.668	β-Gal*p*(1)	H-3 @ 4.020
		β-Gal*p*(1)	H-4 @ 4.215
		β-Gal*p*(1)	H-2 @ 3.970*
β-Gal*p*(1) H-1	H-2 @ {3.960}	GlcNAc-ol	H-6′ @ 4.182
	H-4 @ {4.215}	GlcNAc-ol	H-6′ @ 3.880
	H-5 @ 3.789†		
β-Gal*p*(4) H-1	H-3 @ 3.683	β-Gal*f*	H-2 @ 4.441
	H-5 @ 3.713†	β-Gal*f*	H-3 @ 4.271

{} Indicates a t.o.c.s.y. peak.
* Arises from sequential r.o.e.s.y. and t.o.c.s.y. transfer.
† Assignment tentative.
‡ Referencing error of 0.014 p.p.m. compared with assignment spectra.

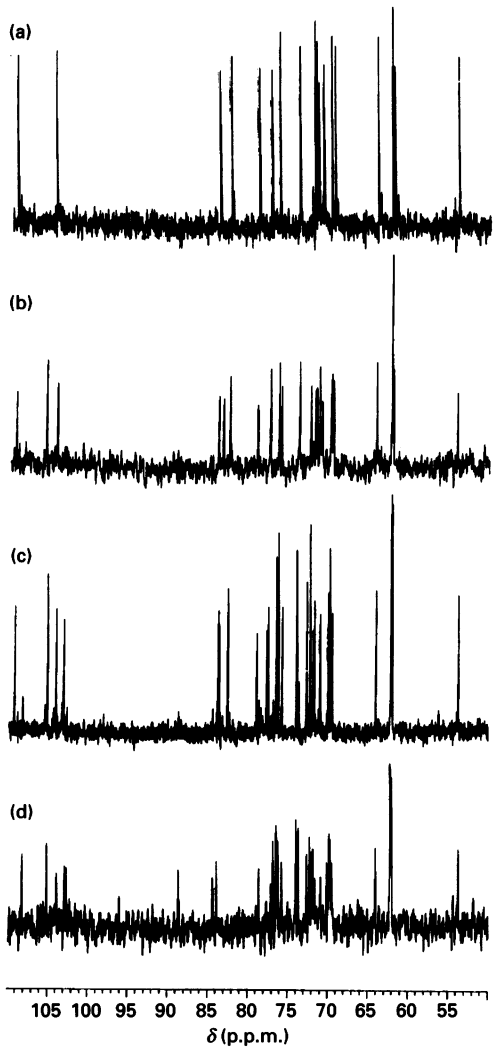

Figure 5 ^{13}C-n.m.r. spectroscopy of the oligosaccharide alditols of *T. cruzi* 38/43 kDa glycoproteins

Partial 125 MHz ^{13}C-n.m.r. spectra of (**a**) disaccharide alditol, (**b**) trisaccharide alditol, (**c**) tetrasaccharide alditol and (**d**) pentasaccharide alditol. Experimental conditions were as described in the Experimental section.

Table 4 Structures of *O*-glycosidically linked GlcNAc-bound oligosaccharides from 38/43 kDa glycoproteins of *T. cruzi* released by reductive β-elimination

Oligosaccharide	Structure
Disaccharide-alditol	β-Gal*f*-(1→4) GlcNAc-ol β-Gal*p*-(1→6) (1)
Trisaccharide-alditol	β-Gal*f*-(1→4) GlcNAc-ol β-Gal*p*-(1→3)-β-Gal*p*-(1→6) (2) (1)
Tetrasaccharide-alditol	β-Gal*f*-(1→4) β-Gal*p*-(1→3) GlcNAc-ol (2) β-Gal*p*-(1→6) (1) β-Gal*p*-(1→2) (3)
Pentasaccharide-alditol	β-Gal*p*-(1→2)-β-Gal*f*-(1→4) (4) β-Gal*p*-(1→3) GlcNAc-ol (2) β-Gal*p*-(1→6) (1) β-Gal*p*-(1→2) (3)

the β-Gal*p*(1) spin system, and weak r.o.e.s.y. correlations between the β-Gal*p*(3) H-1 and β-Gal*p*(1) H-3 and between β-Gal*p*(2) H-1 and β-Gal*p*(1) H-3 were ascribed to sequential r.o.e.s.y. and t.o.c.s.y. transfers (Table 3). A nuclear Overhauser enhancement (n.O.e.) was observed between the β-Gal*p*(1) H-1 and the β-Gal*f* H-1. The β-Gal*p*(1) H-1 showed other inter-residue n.O.e.s to resonances at 4.164 and 3.850 p.p.m., assigned as GlcNAc-ol H-6 and H-6′ respectively (Table 3). The β-Gal*f* H-1 showed inter-residue n.O.e.s to resonances at 3.825, 3.927 and 4.046 p.p.m., assigned as GlcNAc-ol H-4, H-3 and H-5. In the carbon spectra (Figure 5 and Table 2) the resonance of C-2 of the β-Gal*p* residue linked to the GlcNAc-ol residue was shifted 6.5 p.p.m. downfield compared with its position in the tri-saccharide alditol. All of these data are consistent with a Gal*p*β1-3(Gal*p*β1-2)Gal*p*β1 substructure.

In the pentasaccharide alditol the β-Gal*f* anomeric and many of the ring proton resonances were shifted downfield relative to those in the tetrasaccharide. Furthermore an additional terminal β-Gal*p* spin system was present (Figure 4 and Table 2). The r.o.e.s.y. spectrum showed inter-residue n.O.e.s between the anomeric resonance of the additional terminal β-Gal*p* H-1 and the βGal*f* H-2 and H-3 resonances (Table 3). These data are consistent with replacement of the single β-Gal*f* residue with a Gal*p*β1-2 (or β1-3) Gal*f*β-1 structure. Confirmation of the linkage position was achieved by means of a 2D′H[^{13}C]-h.m.q.c. spectrum. The chemical shift of the β-Gal*f* C-2 in this fraction is approximately 6 p.p.m. downfield relative to its position in the tetrasaccharide alditol spectrum, and the C-1 and C-3 resonances are moved slightly upfield (0.9 and 0.5 p.p.m. respectively) (Figure 5). This pattern is typical of glycosylation on the O-2 of the β-Gal*f* residue. An additional β-Gal*f* spin system in the spectrum of disaccharide alditol was observed, possibly arising from contamination with the monosaccharide alditol Gal*f*β1-4GlcNAc-ol. The spectra of the other oligosaccharide alditols contained minor low-field resonances close in chemical shift to that of the -2Gal*f*β1-residue (observed in pentasaccharide alditol), suggesting that a parallel series of compounds was present, for instance Gal*p*β1-2Gal*f*β1-6(Gal*p*β1-2Gal*f*1-4)-GlcNAc-ol contaminating the tetrasaccharide alditol preparation (Figures 4 and 5). Table 4 summarizes the structures of the major *O*-GlcNAc-linked oligosaccharides from the 38/43 kDa glycoproteins of *T. cruzi*.

DISCUSSION

In the present study we have established that the 38/43 kDa glycoproteins expressed at the cell surface of epimastigote forms of *T. cruzi* (G-strain) function as the main sialic acid acceptor in the trans-sialidase reaction (Previato et al., 1985), and we have characterized five major O-linked carbohydrate chains from these highly glycosylated molecules. The oligosaccharides are linked to threonine and/or serine via *N*-acetylglucosamine units (*O*-GlcNAc linked); however, about 20% of the *O*-GlcNAc-linked residues appear not to be substituted by additional sugar units. So far, only single *O*-GlcNAc-linked units have been found in a variety of proteins of eukaryotic cells, from yeast to man (Haltiwanger et al., 1992). These proteins are distributed preferentially in the cytoplasm and nucleus (Kearse and Hart, 1991) and are believed to play an important role in the regulation of fundamental cellular processes. Thus the O-glycosidically linked saccharide occupies sites that are similar to those recognized by serine/threonine kinases; it may also specifically regulate proteolysis, mediate protein–protein interaction and be involved in nuclear targeting (Schnidler et al., 1987; Rogers et al., 1986).

In parasitic organisms, glycoproteins containing single *O*-GlcNAc-linked units have been reported in *Trypanosoma brucei* (Haltiwanger et al., 1992), *Schistosoma mansoni* (Nyame et al., 1987) and *Plasmodium falciparum* (Dieckmann-Schuppert et al., 1993). In the last example, the authors suggested that GlcNAc at the reducing end can be elongated to form longer O-glycosidic oligosaccharides. In *T. cruzi* epimastigotes, most of the *O*-GlcNAc linked is further substituted forming oligosaccharide chains of between three and six units (G-strain) and two and four units (Y-strain) (J. O Previato, C. Jones, R. Wait and L. Mendonça-Previato, unpublished work). These oligosaccharides are acceptors for sialic acid incorporation, and therefore, in the native glycoprotein, a proportion of the O-linked chains will be acidic and therefore constitute a target for appropriate lectins and antibodies (Previato et al., 1985; Schenkman et al., 1991). As previously reported, glycoconjugates from cell-derived *T. cruzi* trypomastigotes contain sialylated epitopes (Ssp3) which are recognized by monoclonal antibodies, and represent important sites for parasite attachment and invasion of the host cell (Schenkman et al., 1991). Invasion of host cells by metacyclic trypomastigotes is also mediated by sialylated glycoproteins (Ruiz et al., 1993; Schenkman et al., 1993); these 35/50 kDa species are presumably analogous to the 38/43 kDa glycoproteins of epimastigotes, described in the present study. Adhesion to host cells is thus likely to be among the functions of the O-linked glycans of these latter molecules, even though epimastigote forms do not progress beyond adhesion to invasion. The role of the short O-linked GlcNAc-containing oligosaccharides and the single GlcNAc units is still unclear. The novel structures of O-linked oligosaccharides of epimastigotes include double substitution of *O*-GlcNAc-linked units. It is particularly interesting that these branched structures are dissimilar in two different

strains of *T. cruzi*. The substitution of GlcNAc by both Gal*f* and Gal*p* units observed in the G-strain does not occur in the Y-strain. In the latter, the major *O*-GlcNAc-linked oligosaccharides had a core structure containing GlcNAc units substituted on either O-3 or O-4 positions by Gal*p* residues (J. O. Previato, C. Jones, R. Wait and L. Mendonça Previato, unpublished work).

The antigenicity of the O-glycosidically linked chains of *T. cruzi* glycoconjugates is suggested by their recognition by monoclonal antibodies (Schenkman et al., 1993). Recent results (I. C. Almeida, L. R. Travassos and M. A. J. Ferguson, unpublished work) showed that glycoproteins from cell-derived trypomastigotes also contain *O*-GlcNAc-linked oligosaccharides which react with lytic anti-α-galactosyl antibodies from patients with Chagas' disease (Almeida et al., 1991). In the case of the epimastigote glycoproteins, the presence of β-D-Gal*f* units in the G-strain can represent an important site for antibody recognition as shown previously in another glycoconjugate from *T. cruzi*, the so-called lipopeptidophosphoglycan, using rabbit (Mendonça-Previato et al., 1983; Xavier et al., 1991) and human antisera (Schnaidman et al., 1986).

The O-linked structures of epimastigote glycoproteins and the similar molecules from trypomastigotes constitute a group of mucin-like glycoconjugates comparable with the sialoglycoconjugates of metacyclic trypomastigotes (Schenkman et al., 1993). Thus they have abundant O-linked chains, usually containing sialic acid and galactose and are linked to threonine and/or serine mainly via *N*-acetylglucosamine rather than *N*-acetylgalactosamine.

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