Biochem. J. (1983) **213**, 313–319
Printed in Great Britain

Studies on the structure of a phosphoglycoprotein from the parasitic protozoan *Trypanosoma cruzi*

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(*Received 10 January 1983/Accepted 25 March 1983*)

A glycoprotein (GP72) has been isolated from *Trypanosoma cruzi* and found to contain 41% protein, 49% carbohydrate and 10% phosphate. All phosphate was covalently attached to the carbohydrate which contained the following sugars: ribose, xylose, fucose, galactose, mannose, glucose and glucosamine. The carbohydrate side chains were linked to protein by fucose, xylose and *N*-acetylglucosamine; 50% of the total *N*-acetylglucosamine was involved in glycoprotein linkages. Two classes of carbohydrate side chains were detected. One class comprised 15% of the total carbohydrate and contained glucosamine, mannose and galactose; some of these chains were phosphorylated. The other class comprised 85% of the total carbohydrate and contained xylose, ribose, fucose, galactose, mannose, glucosamine and phosphate; these chains were antigenic and reacted with a monoclonal antibody with specificity for the whole glycoprotein.

Trypanosoma cruzi is a protozoan parasite which is the causative agent of Chagas' disease, a chronic debilitating disease which is estimated to afflict between 10 and 12 million people in South and Central America (Brener, 1982). The protozoan has a complex life cycle involving blood-sucking reduviid bugs and mammals. It multiplies in the insect gut as an epimastigote form and is spread as a non-dividing metacyclic trypomastigote from the insect faeces by contamination of wounds produced by the blood-sucking activity of the vector. In the mammalian host, *T. cruzi* multiplies intracellularly in the amastigote form and is subsequently released into the bloodstream as a non-dividing trypomastigote (Brener, 1973). A glycoprotein specific for the insect stages of *T. cruzi* has been identified (Snary *et al.*, 1981; Nogueira *et al.*, 1981) and studies using a monoclonal antibody specific for this glycoprotein have led to the hypothesis that the glycoprotein is involved in the control of morphogenesis of *T. cruzi*, possibly by interaction of the parasite with specific insect gut lectins (Sher & Snary, 1982). The glycoprotein has an apparent M_r of 72000 and has been isolated by monoclonal antibody affinity chromatography; mice immunized with this purified glycoprotein were protected from an insect-derived metacyclic trypomastigote challenge but not against a blood trypomastigote challenge (Snary, 1983). This glycoprotein has an unusual carbohydrate composition comprising mannose, glucose, galactose, fucose, xylose and ribose (Snary *et al.*, 1981). Further studies on the structure of this glycoprotein are described in the present paper.

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Experimental

Materials

Sugars and their alditols were bought from Sigma with the exception of fucitol and *N*-acetylglucosaminitol which were prepared by reduction with $NaBH_4$.

Glycoprotein preparation and parasite cultivation

A clone of the Y strain of *T. cruzi* (Wel Tryp Y2C1) was grown in the epimastigote form in Boné and Parent's medium containing 5% (v/v) rabbit serum, penicillin and streptomycin as described previously (Snary *et al.*, 1981). The procedures for the production of monoclonal antibody and its utilization for the purification of the glycoprotein by affinity chromatography are also given by Snary *et al.* (1981). It should be emphasized that although this glycoprotein (GP72) is a major antigenic component of the cell surface of *T. cruzi*, it only

represents 0.04% of the whole cell protein and that 3×10^{12} organisms (equivalent to 30 litres of culture) are required to produce 1 mg of glycoprotein.

Analytical methods

The amino acid content of samples of glycoprotein was determined after hydrolysis in 3 M-toluene-*p*-sulphonic acid at 110°C for 24h under N_2 (Liu, 1972) and amino sugars after hydrolysis in 3 M-toluene-*p*-sulphonic acid at 100°C for 24h under N_2 (Allen & Neuberger, 1975). Analyses were done on a Locarte Mini amino acid analyser with the buffer systems which have been described elsewhere (Allen *et al.*, 1976). *p*-Fluorophenylalanine and norleucine were used as internal standards. Neutral sugars were measured by g.l.c. on a Perkin–Elmer F33 fitted wtih flame ionization detectors after methanolysis and trimethylsilylation of the glycoprotein (Chambers & Clamp, 1971). The columns were packed with 8.5% (w/w) SE30 as the liquid phase on a support of Diatomite C.A.W.; N_2 was the gaseous phase. Mannitol was used as an internal standard. Phosphorus was determined by the method of Bartlett (1959).

Treatment of the glycoprotein with 0.5 M-NaOH

The glycoprotein (2.7mg) was suspended in 1.5ml of 0.5 M-NaOH which contained 1 mM-$CaCl_2$ and was incubated at 40°C for 120h. The reaction mixture was then cooled and centrifuged at 500 *g* for 5min to remove the remaining insoluble protein. On analysis the insoluble protein was found to have a similar amino acid composition to the original glycoprotein but had no significant amounts of carbohydrate. The supernatant was neutralized by the addition of 6 M-HCl and applied to a column (20cm × 0.9cm) of Bio-Gel P-2 and eluted with water.

Fractionation of oligosaccharides

The desalted fraction from the previous step was freeze-dried, dissolved in 0.4ml of water and fractionated on a QAE-Sephadex (A-25) column (4cm × 0.5cm) which had been equilibrated with 2 mM-ethanolamine. Ethanolamine was used in preference to Tris (Varki & Kornfeld, 1980) because it is more easily removed from solution by evaporation and, unlike Tris, it does not produce peaks in the carbohydrate region of the g.l.c. chromatogram. The column was eluted firstly with 2 mM-ethanolamine (16ml), then a gradient from 2 mM-ethanolamine to 2 mM-ethanolamine containing 0.6 M-NaCl. Fractions (2ml) were collected and assayed for their carbohydrate and phosphate content.

Radioactive labelling of the glycoprotein linkage sugars

The glycoprotein (150μg) was incubated in 40μl of a solution of 0.2 M-NaOH/1 mM-$CaCl_2$/1 M-NaB^3H_4 (1mCi/μmol) for 120h at 40°C. The reaction mixture was then adjusted to pH 5.0 by the addition of glacial acetic acid, and centrifuged at 500 *g* for 5min. The supernatant was applied to a Bio-Gel P-2 column which was eluted with water (see above) to remove salts and low (<200) molecular weight components which had been tritiated. The oligosaccharide fraction which was eluted at V_0 was then concentrated by freeze-drying and re-chromatographed on the same column. The labelled oligosaccharide fraction was then divided into appropriate aliquots for further investigation.

Determination of the neutral sugars involved in glycoprotein linkages

An aliquot (10μg) of the labelled polysaccharide (see above) was subjected to acid hydrolysis (2 M-HCl at 100°C for 3h under N_2). The hydrolysate was then dried over P_2O_5 and NaOH, redissolved in 0.5ml of 10mM-HCl and applied to a column (4cm × 0.5cm) of Dowex 50 X2 (H^+ form) and eluted with 10mM-HCl (5ml). The eluate was collected and concentrated. This step removed the majority of the labelled amino acids and peptides together with any labelled hexosamines, which accounted for about 50% of the total radioactivity that was applied.

T.l.c. was performed on cellulose-coated plates (20cm × 20cm; Merck) which were developed twice with ethyl acetate/pyridine/water (10:4:3, by vol.). ^{3}H-labelled sample lanes were scraped in 3mm strips and the scrapings were shaken with 0.5ml of water for 30min; then 8ml of Aquasol-2 was added and radioactivity was determined by using a variable quench compensating programme. Positions of authentic sugar alcohol standards were determined by subsequent $AgNO_3$/NaOH staining of adjacent lanes (Bailey, 1969).

Determination of amino sugars involved in glycoprotein linkages

The same method previously described for neutral sugars was employed, except that the Dowex 50 column step was omitted.

Determination of O- and N-glycosidic N-acetyl glucosamine

The method of Mega & Ikenaka (1982) was used. Samples (450μg) were repeatedly dried *in vacuo* over P_2O_5 and methanolysed in dry 1 M-HCl in methanol at 100°C for 24h under N_2. The methanolysate was filtered and the filtrate and washings dried under N_2. The relative proportions of glucosamine derivatives was determined on an amino acid analyser eluted with a standard pH 5.28 buffer.

Radioimmunoassay

The radioimmunoassay used polyvinylchloride multiwell plates coated with purified GP72 and bound antibody was detected by an ^{125}I-labelled $(Fab')_2$ fragment of immunopurified rabbit anti-mouse immunoglobulin (Snary, 1983). Monoclonal antibody WIC 29.26 at a dilution of 1:10000 was used in inhibition studies; the antibody was incubated for 30min at 25°C before assay with aliquots of the fractions eluted from the QAE-Sephadex fractionation of alkali-released carbohydrate.

Results

Analysis and characterization of sugars

The analysis of GP72 is given in Table 1. The values are essentially those that we reported previously (Snary *et al.*, 1981) with the exception of the phosphate that has not previously been reported. The ribose and xylose peaks were recognized by their characteristic retention times relative to the internal standard and their patterns of peaks produced on methanolysis. The identity of the ribose was confirmed by co-chromatography with an authentic standard using a number of different programmes.

Since the occurrence of ribose in the glycoprotein was unexpected, the possibility that the glycoprotein contained either contaminating RNA or was an ADP-ribosylated protein was considered. To test this hypothesis GP72 (250μg) was incubated with 13 units of ribonuclease A (Worthington) for 12h at 37°C in 100mM-sodium acetate buffer, pH5.0, containing 0.05% (v/v) Renex 30 (Honeywell Atlas, Carshalton, Surrey, U.K.). (The ribonuclease had previously been shown to retain 80% of its activity under these conditions.) After extensive dialysis and re-analysis the composition of GP72 was shown to be unchanged.

Since any nucleotide polymer should have an equimolar ratio of base to ribose, the u.v.-absorption profile should be characteristic of RNA with a high absorption at 260nm. A u.v.-absorption spectrum of GP72 revealed that there was no pronounced peak at 260nm. Furthermore, as discussed below, the ribose and phosphate were contained within alkali-stable structures. All of these observations rule out the presence of contaminating RNA.

Table 1. *Composition of GP72*

	Proportion (%) by weight	Residues/100 amino acids	
Amino acids*	41	Asx	8.4
		Thr	9.3
		Ser	12.6
		Glx	8.5
		Pro	5.0
		Gly	6.8
		Ala	6.4
		½Cys	1.2
		Val	9.4
		Met	1.5
		Ile	5.2
		Leu	9.9
		Tyr	3.0
		Phe	2.2
		His	3.3
		Lys	3.4
		Arg	4.0
Sugars	49	Rib	7.7
		Fuc	10.8
		Xyl	19.1
		Man	12.3
		Gal	23.3
		Glc	2.0
		NeuAc	0
		GlcN	4.4
		GalN	0
Phosphate	10		26.0

* Data taken from Snary *et al.* (1981). Trp was not determined; Hyl and Hyp were not detected.

Carbohydrate release and oligosaccharide fractionation

Oligosaccharide side chains were released from the glycoprotein by treatment with 0.5M-NaOH and desalted on a column of Bio-Gel P-2. The elution pattern is shown in Fig. 1 and analysis of the material which was eluted in the void volume showed that it contained all the carbohydrate and phosphorus of the original glycoprotein together with some peptide material that had been released

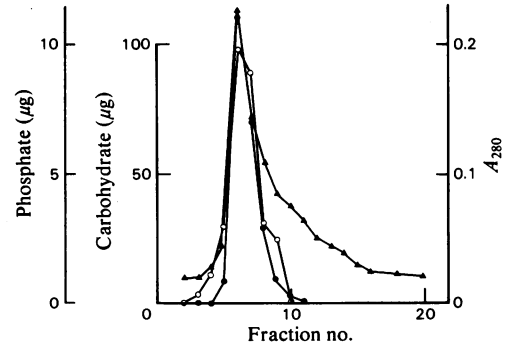

Fig. 1. *Gel filtration of alkali-treated GP72 on Bio-Gel P-2*

For experimental details see the text; O, carbohydrate; ▲, protein (A_{280}); ●, phosphate.

from the glycoprotein by alkaline hydrolysis (no carbohydrate was found in the insoluble protein residue left after alkali treatment). The conditions which were used should have been sufficient to cleave all carbohydrate linkages involving serine, threonine and asparagine (Ogata & Lloyd, 1982). There was no carbohydrate eluted in the positions expected for mono- to tetrasaccharides, nor was there any detectable phosphate in the included volume. We therefore concluded that all the carbohydrate of the original glycoprotein, including ribose, is associated with oligosaccharides which contain at least five sugars. In addition it is noteworthy that the phosphate is associated with the oligosaccharide fraction and that it is not attached directly to the hydroxyamino acids threonine and serine, since such linkages are known to be alkali-labile especially in the presence of Ca^{2+} (Whitaker & Feeney, 1977).

The carbohydrate-containing fractions from the Bio-Gel P-2 column were subsequently fractionated on a QAE-Sephadex column. The elution profile (Fig. 2) shows five distinct peaks of carbohydrate-containing material and the compositions of the fractions are given in Table 2. The first two peaks (Ia and Ib) had a very similar carbohydrate composition, lacked phosphate and are considered to be essentially the same material which had been separated by non-specific adsorption, probably caused by the low ionic strength of the initial buffer. The material from fraction II resembles that of I except that it contains phosphate and lower levels of glucosamine. Peaks IIa and IIb contain 85% of the total carbohydrate of the original glycoprotein and 83% of the total phosphate. They also contain both the pentoses and the methyl pentose as well as mannose, galactose and glucosamine.

Determination of the linkage sugars of the glycoprotein

The alkaline treatment of the glycoprotein was repeated in the presence of NaB^3H_4. Under these conditions the oligosaccharides become labelled at their reducing ends by the conversion of the former linking sugar to the corresponding alcohol. Identification of the ^3H-labelled alditols was by t.l.c. on cellulose plates. The system gave good resolution of fucitol, ribitol and xylitol and also separated them from the hexitols. The distribution of radioactivity in the chromatogram is shown in Fig. 3. There are radioactive peaks which are coincident with markers for xylitol and fucitol as well as material near the origin of the chromatogram which does not co-chromatograph with any alditol. This result implies that both xylose and fucose are involved in linkages to the protein, presumably to serine and threonine residues since these are known to be alkali-labile (Marshall & Neuberger, 1970). It can also be said that ribose, galactose, glucose and mannose are not involved in any alkali-labile linkages to the polypeptide chain.

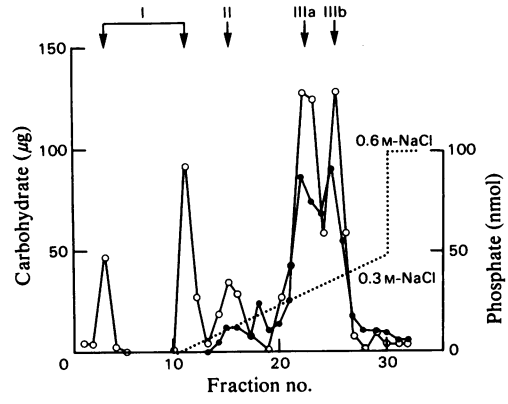

Fig. 2. *Fractionation of alkali-released oligosaccharides on QAE-Sephadex A-25*

For experimental details see the text; the column was eluted with a gradient from 2 mM-ethanolamine to 2 mM-ethanolamine/0.3 M-NaCl and then washed with 2 mM-ethanolamine/0.6 M-NaCl. O, Carbohydrate; ●, phosphate; ····, NaCl gradient.

Table 2. *Carbohydrate composition of peaks recovered from QAE-Sephadex fractionation*

Component	Peak	I	II	IIIa	IIIb
	[NaCl] required for elution (mM)	0	75	165	205
	Total carbohydrate (%)	5	10	47	38
		Composition (mol %)			
Rib		0	0	6	9
Fuc		0	0	8	14
Xyl		0	0	19	24
Man		72	71	16	5
Gal		12	16	23	23
GlcN		16	4	6	3
Phosphate		0	9	23	22

Determination of the amino sugars involved in glycoprotein linkages

In the investigation of the neutral sugars (see above), any ^{3}H-labelled glucosaminitol that would have been produced by the cleavage of chains linked through *N*-acetylglucosamine would not have been detected because glucosaminitol would be removed by the Dowex 50 (H^+) under the conditions used. Preliminary experiments to demonstrate the presence of [^{3}H]glucosaminitol and its *N*-acetyl derivative after acetylation did indicate that labelled glucosaminitol was present in small amounts, but contamination by other amino compounds from the hydrolysate made the unequivocal interpretation of the chromatograms difficult. The ^{3}H-labelled oligosaccharides were therefore chromatographed on QAE-Sephadex in the manner described for the unlabelled oligosaccharides (see above) and a similar profile was obtained. The material corresponding to fraction I (Fig. 2) was hydrolysed and co-chromatographed with glucosaminitol as before. In this case, the only radioactive compound on the chromatogram was glucosaminitol, showing unequivocally that glucosamine, presumably as *N*-acetylglucosamine, is involved as a linkage sugar in the glycoprotein.

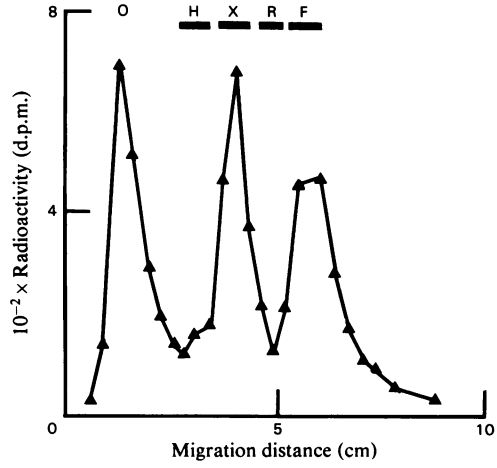

Fig. 3. *Separation of ^{3}H-labelled neutral sugar-alcohols on cellulose t.l.c. plates*

Details are given in the text. O, origin; H, hexitols; X, xylitol; R, ribitol; F, fucitol. Bars under these letters represent the position to which standard sugars ran on the t.l.c. plates.

Determination of N- and O-glycosidic N-acetylglucosamine

The ratio of *O*-glycosidic to *N*-glycosidic *N*-acetylglucosamine was estimated by the method of Mega & Ikenaka (1982). This method discriminates between *N*- and *O*-glycosidic *N*-acetylglucosamine by the relative ratios of their methanolysis products: glucosamine, methyl-α-glucosamine and methyl-β-glucosamine. This is because an *N*-linked *N*-acetylglucosamine yields predominantly glucosamine whereas an *O*-linked *N*-acetylglucosamine yields predominantly methyl-α-glucosamine; thus the ratio of these two products can be used to determine the relative proportions of *O*- and *N*-linked *N*-acetylglucosamine (Table 3). This technique was investigated using as a model compound a soya-bean agglutinin glycopeptide which contained one '*N*-linked' and one '*O*-linked' *N*-acetylglucosamine in its high-mannose asparagine-linked carbohydrate side chain (Lis & Sharon, 1978). Preliminary experiments showed that yields were dependent upon relatively minor temperature changes during methanolysis, but that results were consistent provided that calibration curves were drawn for Asn-GlcNAc [*N*-(L-asparto-4-oyl)-2-acetamido-2-deoxy-β-D-glucosylamine] and *N*-acetylglucosamine standards which had been methanolysed at the same time and under identical conditions to the unknown samples. Under these conditions, close to theoretical values were given for the soya bean agglutinin glycopeptide. Application of this method to GP72 produced the values shown in Table 3; conversion of the methyl-α-glucosamine:glucosamine ratio to an *O*- to *N*-glycosidic link ratio from a graph drawn with data calculated for standard compounds gave a value of 1.0:1 for *O*- to *N*-linkages for GP72.

Table 3. *Analysis of methanolysis products of GP72*

Sample	Amount (peak areas in arbitrary units) of: GlcN	Me-β-GlcN	Me-α-GlcN	Ratio of Me-α-GlcN/ GlcN
GlcNAc (1 mmol)	0.36	0.75	2.84	7.89
Asn-GlcNAc (1 mmol)	1.26	–	0.14	0.11
GP72	1.31	1.23	2.43	1.84
Theoretical value for GlcNAc (1 mmol) + Asn-GlcNAc (1 mmol)	1.62	0.75	2.98	1.85

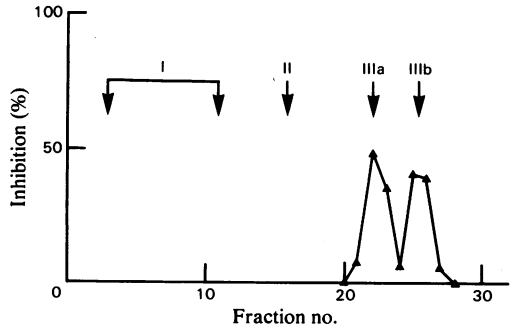

Fig. 4. *Antigenic activity of alkali-released oligosaccharides of GP72*

Alkali-treated GP72 was fractionated on QAE-Sephadex A-25 and the fractionated material was used to inhibit the binding of monoclonal antibody WIC 29.26 to intact GP72 in a solid-phase radioimmunoassay; details are given in the text. I, II, IIIa and IIIb represent the elution positions of these peaks (see Fig. 2).

Antigenicity of carbohydrate

The monoclonal antibody which had been used to isolate GP72 was shown by inhibition of radioimmunoassay to react with the carbohydrate side chains of the antigen. Inhibition of antibody binding was given only by the pentose-rich peaks (fractions IIIa and IIIb) from the QAE-Sephadex fractionation (Fig. 4). Even at concentrations 400-fold higher than those which gave complete inhibition with the pentose-rich peaks no inhibition was given by other fractions. The carbohydrate specificity of the monoclonal antibodies was confirmed by the ability of 10mM-periodate to abolish the antigen–antibody interaction (results not shown); no inhibition of binding by monosaccharides was however demonstrable even at concentrations of sugar as high as 100mM.

Discussion

The M_r 72000 glycoprotein is a membrane component of *T. cruzi* which has been purified by affinity chromatography using a monoclonal antibody. It shows a number of features in its composition and structure which are very unusual, including the presence of ribose. As far as we are aware, ribose has not previously been shown to be present in a glycoprotein, except for the ADP-ribosylated proteins (Hayaishi & Ueda, 1977). The identification was based on the characteristic position and pattern of peaks on g.l.c. following methanolysis and trimethylsilylation and co-chromatography with an authentic ribose sample. The small quantities of the glycoprotein that we were able to isolate have so far precluded the isolation of ribose from hydrolysates to allow its characterization and to determine whether it is the natural D-stereoisomer or the L-stereoisomer. The presence of the 'unnatural' stereoisomer of a pentose is a possibility, since a related trypanosome, *Crithidia fasciculata*, has been shown to have polysaccharides containing D-arabinose (Gorin *et al.*, 1979) rather than L-arabinose that occurs in plant glycoproteins and polysaccharides.

An unusual feature of this glycoprotein is that it has three different sugars that are involved in linkages to amino acids of the polypeptide chain; these are *N*-acetylglucosamine, xylose and fucose. *N*-Acetylglucosamine when linked to proteins in animal, plant and protozoal glycoproteins, including trypanosomal glycoproteins (Holder & Cross, 1981), is invariably linked by a *N*-glycosylamine linkage to asparagine. We have shown that 50% of the glucosamine residues are probably *N*-linked and this would presumably be to asparagine. Xylose residues in this glycoprotein are presumably linked to serine or threonine, since the oligosaccharides linked by this sugar were released by alkaline treatment and hydroxyproline and hydroxylysine were not found in this protein. Linkages between xylose and serine or threonine are known in the proteoglycans of higher animals (Rodén & Horowitz, 1978), in red algae (Heaney-Kieras *et al.*, 1977) and in maize root cap slime (Green & Northcote, 1978) but have not previously been reported in protozoal glycoproteins.

Fucose is usually found in mammalian glycoproteins as a non-reducing terminal sugar and its occurrence as a linkage sugar in this glycoprotein is unexpected; because of its alkali-lability it is presumably linked to serine or threonine. Linkages between fucose and serine or threonine have been reported for glycosides isolated from mammalian urine (Klinger *et al.*, 1981).

Further unusual features of GP72 are the very high (10%) proportion of phosphate in the molecule and the observation that all the phosphate is attached to sugars and in particular those of the pentose-rich side chains. These same pentose-rich chains are the sites in the molecule which are recognized by the monoclonal antibody. The high degree of antigenicity is perhaps to be expected as the high phosphate content, presence of xylose and ribose together with the linkages to fucose and xylose are all features which would be recognized as foreign by a mammalian immune system. Mammalian phosphorylated glycoproteins have been shown to contain mannose 6-phosphate (Varki & Kornfeld, 1980) but the ratio of phosphate to mannose of 4.4:1 in peak IIIb precludes this sugar from being the only phosphorylated sugar. The presence of high levels of phosphate in this glycoprotein could invalidate the molecular weights estimated by sodium dodecyl sulphate/polyacryl-

amide-gel electrophoresis. The phosphate will increase the negative charge of the glycoprotein, which ought to migrate relatively faster than the standard proteins; the estimated M_r of 72000 is therefore probably a minimum value and the actual value may be substantially greater.

It would appear that the carbohydrate side chains of GP72 fall into two basic types (Table 2). One of these contains all of the pentoses and methylpentoses of the molecule, is highly phosphorylated and constitutes approx. 85% of the carbohydrate carried by GP72. Two sugars, fucose and xylose, are probably involved in protein–carbohydrate linkages, but it remains to be determined whether each linkage sugar is associated with a distinct structure such as the separable peaks IIIa and IIIb, or whether the difference between a protein-to fucose or -xylose linkage does not affect the subsequent carbohydrate side chain structures produced, in which case peaks IIIa and IIIb will both contain fucose- and xylose-to-protein linkages. The remaining carbohydrate is mannose-rich and is probably linked through glucosamine to asparagine in the polypeptide. Peak I fits this model chain structure well, whereas Peak II is phosphorylated and contains relatively low levels of glucosamine. If a chitobiose-to-asparagine link is invoked then this chain must be extremely large (>30 mannose residues) and heavily phosphorylated. It remains to be seen whether this or some alternative structure is found; one alternative could be the serine–phosphodiester–glucosamine linkage found in slime moulds (Gustafson & Milner, 1980).

The carbohydrate structure of this glycoprotein is clearly unusual if not unique, and further work is essential before a detailed structure is obtained. A knowledge of this structure may be essential however if we are to understand how the glycoprotein may function at the molecular level to control the morphogenesis of *T. cruzi* within its insect vector.

We thank Mr. M. A. Smith for technical assistance. M. A. J. F. thanks the S.E.R.C. for CASE Studentship.

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