Complete Structure of the Glycan of Lipopeptidophosphoglycan from Trypanosoma cruzi Epimastigotes*

Rosa M. de Lederkremer, Carlos Lima, and Maria I. Ramirez
From the Departamento de Quimica Organica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentina

Michael A. J. Ferguson, Steve W. Homans, and Jane Thomas-Oates‡
From the Department of Biochemistry, University of Dundee, Dundee DD1 4HN, United Kingdom

The lipopeptidophosphoglycan is the major cell surface glycoconjugate of the epimastigote forms of the parasitic protozoan Trypanosoma cruzi. A detailed partial structure for this molecule has been reported (Previo, J. O., Gorin, P. A. J., Mazurek, M., Xavier, M. T., Fournet, B., Wieruszewski, J. M., and Mendonca-Previo, L. (1990) J. Biol. Chem. 265, 2518-2526). In this study, we complete the primary structure assignments and describe the microheterogeneity found in the lipopeptidophosphoglycan glycan, using a combination of 2H and 31P NMR, fast atom bombardment mass spectrometry, methylation linkage analysis, and exoglycosidase sequencing. The lipopeptidophosphoglycan is a glycosylated inositol-phosphoceramide with striking homology to glycosylphosphatidylinositol membrane anchors found attached to a wide variety of plasma membrane proteins throughout the eukaryotes.

The parasitic protozoan Trypanosoma cruzi is the causative agent of Chagas' disease (South American trypanosomiasis). The organism undergoes a complex life cycle between a wide variety of mammalian hosts and biting insect vectors (reduvid bugs). In the infected mammal, the parasite exists as intracellular dividing amastigote forms in tissues such as smooth and cardiac muscle. These forms give rise to extracellular nondividing bloodstream trypomastigote forms that spread the infection. The ingestion of trypomastigotes by the insect vector results in the differentiation of the parasite to the dividing epimastigote form, which efficiently colonizes the insect midgut. Migration of parasites to the insect hindgut results in their differentiation to metacyclic trypomastigote forms that are adapted for transmission to a mammalian host via fecal contamination of fresh wounds or mucous membranes.

The lipopeptidophosphoglycan (LPPG) is the most abundant glycoconjugate of the epimastigote form of the parasite; a typical yield of LPPG is about 100 mg extracted from 2 x 10^12 cells (Lederkremer et al., 1990). Assuming a molecular weight of around 1890 (Previo et al. (1990) and this study) this suggests a minimum copy number of around 1.5 x 10^7 LPPG molecules/epimastigote. The function of LPPG is unknown, but it appears to be restricted to the epimastigote forms of the parasite (Zingales et al., 1982).

The LPPG fraction contains mannose, galactofuranose, 2-aminoethylphosphonate (AEP), myo-inositol, phosphate, long chain bases, and fatty acids, together with traces of glucose and amino acids (Lederkremer et al., 1978; Ferguson et al., 1981, 1985; Lederkremer et al., 1986). The lipid component is an inositol-phosphoceramide containing mainly palmitoylsphinganine, palmitoylsphingosine, and lignoceroylsphinganine. The ceramide can be released by phosphorylceramidase (PI)-specific phospholipase C and the glycan chain is attached to the inositol ring via a glycosidic linkage to a non-N-acetylated glucosamine (GlcN) residue (Lederkremer et al., 1990). These two latter features indicate that it is closely related to the glycosylphosphatidylinositol (GPI) membrane anchors common to many cell surface glycoproteins throughout the eukaryotes (reviewed recently by Thomas et al. (1990); Cross, 1990).

A detailed structure of the LPPG glycan was recently reported by Previo et al. (1990). In this paper, we confirm these structural features and complete the glycan structure by supplying (i) the nature of the glycosidic linkage between glucosamine and myo-inositol, (ii) the precise location of both galactofuranose residues, and (iii) the degree of heterogeneity in the glycan structure. The LPPG structure is discussed in the context of general and parasite-specific GPI metabolism.

DISCUSSION

The analysis of the LPPG glycan moiety, generated by PI-specific phospholipase C cleavage of the ceramide lipid, presented some difficult problems. Attempts to fractionate the different glycan species by Dionex carbohydrate high pressure liquid chromatography produced a confusing array of peaks (data not shown) due to the presence of three major glycan species and compounded by the heterogeneity of the inositol phosphorylation state. Deamination and reduction was used to introduce a labeled 2,5-anhydromannitol terminus and to remove the inositol phosphate. However, Dionex high pres-

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‡ Portions of this paper (including "Materials and Methods," "Results," Figs. 1–5, and Tables 1 and 2) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
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Supplemental Material:

Compare the glycan of lipopeptidophosphoglycan from *Trypanosoma cruzi* with that of a known glycan from *Leishmania major*.

**Materials and Methods**

**Parasite culture** and purification of LPPG. *T. cruzi* amastigotes were isolated from the mouse liver, and grown in culture media containing fetal bovine serum. The amastigotes were harvested by centrifugation and washed twice with phosphate-buffered saline (PBS). The purified amastigotes were incubated with 0.4% formaldehyde and 0.02% sodium azide in PBS, and then washed twice with PBS. The glycoprotein was then purified by gel filtration chromatography on a Sephadex G-25 column in PBS. The purified glycoprotein was then subjected to SDS-PAGE and identified by Western blot analysis using anti-LPPG antibodies.

**Preparation of fraction A**. Non-reducing, non-irradiated LPPG from *T. cruzi* amastigotes was subjected to size exclusion chromatography on a Superose 12 column in PBS. The eluted glycoprotein was subjected to SDS-PAGE and stained with Coomassie Blue. The purified glycoprotein was then subjected to N-terminal sequencing using an automated sequencer.

**Preparation of fraction C**. The glycoprotein was subjected to Endo H and T. cruzi amastigotes were treated with 0.5% formaldehyde and 0.02% sodium azide in PBS. The purified glycoprotein was then subjected to SDS-PAGE and stained with Coomassie Blue. The purified glycoprotein was then subjected to N-terminal sequencing using an automated sequencer.

**Preparation of fraction D**: The glycoprotein was subjected to Endo H and T. cruzi amastigotes were treated with 0.5% formaldehyde and 0.02% sodium azide in PBS. The purified glycoprotein was then subjected to SDS-PAGE and stained with Coomassie Blue. The purified glycoprotein was then subjected to N-terminal sequencing using an automated sequencer.

**Preparation of fraction E**: The glycoprotein was subjected to Endo H and T. cruzi amastigotes were treated with 0.5% formaldehyde and 0.02% sodium azide in PBS. The purified glycoprotein was then subjected to SDS-PAGE and stained with Coomassie Blue. The purified glycoprotein was then subjected to N-terminal sequencing using an automated sequencer.

**Preparation of fraction F**: The glycoprotein was subjected to Endo H and T. cruzi amastigotes were treated with 0.5% formaldehyde and 0.02% sodium azide in PBS. The purified glycoprotein was then subjected to SDS-PAGE and stained with Coomassie Blue. The purified glycoprotein was then subjected to N-terminal sequencing using an automated sequencer.
The generation of inositol-1,2-cyclic phosphate is characteristic of the action of bacterial PI-specific phospholipase C enzymes, and subsequent opening of the cyclic phosphate can lead to an additional mixture of inositol 1-phosphate and inositol 2-phosphate. In this case, only one inositol-monophosphate signal is seen in addition to the inositol 1,2-cyclic phosphate in the \( \cdot P \)NMR spectrum. Analysis of a mixture of unsubstituted inositol 1-phosphate and inositol 2-phosphate indicated that the two forms give rise to well resolved signals at 5.28 and 6.06 ppm, respectively. The results indicate that, under the conditions used in this paper, \( B. \) thuringiensis PI-specific phospholipase C (Volwerk et al., 1990) can generate both the 1,2-cyclic phosphate and the 1-phosphate from the LPPG substrate. This result is in agreement with the observations made for \( B. \) cereus PI-specific phospholipase C (Volwerk et al., 1990) that showed that this enzyme has an intrinsic, regio-specific, cyclic phosphodiesterase activity.
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Table 1: Multiplicity linkage composition of fractions A, C, and D

| Multiplicity Linkage | Origin | Fraction A | Fraction C | Fraction D |
|----------------------|--------|------------|------------|------------|
| [A-Galp]α1,6[Galβ1,3](GlcNAc)2PO4 | Untreated | 1.68 | 1.66 | 1.66 |
| [A-Galp]α1,6[Galβ1,3](GlcNAc)2PO4 | Untreated | 1.68 | 1.66 | 1.66 |
| [A-Galp]α1,6[Galβ1,3](GlcNAc)2PO4 | Untreated | 1.68 | 1.66 | 1.66 |
| [A-Galp]α1,6[Galβ1,3](GlcNAc)2PO4 | Untreated | 1.68 | 1.66 | 1.66 |

*This table shows the relative peak areas, with several peaks merged to form the final values.

**Note:** The peak areas have been normalized to 100%.

SC-MS methylation analyses and amino acid sequencing:
The SC-MS methylation analysis of the fraction C which was left after the FAB-MS analysis (Table 2) is entirely consistent with the NMR and FAB-MS assignments of the major structure. This major structure contains two terminal Gal residues and a 2-D-P-D-GlcNAc constituent residue. In addition, the removal of the AEP moiety during the methylation step is indicated by the appearance of the 4-Deoxy-D-Galactose (4Gal) residue in fraction C (Table 2, fraction A, where the GalNAc residue is methylated in the methylation analysis due to its phosphorylated mass). Evidence for the position of the AEP-4Gal residue was obtained from the methylation analysis of methylated and N-acetylated fraction A. The deacetylated, reduced products were characterized by GC-MS analysis, and a complex series of peaks was observed (data not shown). GC-MS linkage analysis of several of the major peaks revealed the novel derivative shown in Figure 4. The mass spectrum indicates that this derivative was derived from an unknown AEP, which in turn was derived from a D-P-D-GlcNAc backbone, and this AEP glycosylates and reduces and reduces.

The linkage analyses of fractions A (Table 2) confirmed the homogeneity of the sample observed by NMR (fraction A), and FAB-MS (Table B). The absence of terminal Man residues shows that all the structures possess non-reducing ends, though the 2-3-D-Galp and 3-3-D-Galp are observed (data not shown). GC-MS analysis of several of the major peaks revealed the novel derivative shown in Figure 4. The mass spectrum indicates that this derivative was derived from an unknown AEP, which in turn was derived from a D-P-D-GlcNAc backbone, and this AEP glycosylates and reduces and reduces.

The FAB-MS analysis of fraction C (Table 2) confirmed the homogeneity of the sample observed by NMR (fraction A), and FAB-MS (Table B). The absence of terminal Man residues shows that all the structures possess non-reducing ends, though the 2-3-D-Galp and 3-3-D-Galp are observed (data not shown). GC-MS analysis of several of the major peaks revealed the novel derivative shown in Figure 4. The mass spectrum indicates that this derivative was derived from an unknown AEP, which in turn was derived from a D-P-D-GlcNAc backbone, and this AEP glycosylates and reduces and reduces.

The FAB-MS analysis of fraction C (Table 2) confirmed the homogeneity of the sample observed by NMR (fraction A), and FAB-MS (Table B). The absence of terminal Man residues shows that all the structures possess non-reducing ends, though the 2-3-D-Galp and 3-3-D-Galp are observed (data not shown). GC-MS analysis of several of the major peaks revealed the novel derivative shown in Figure 4. The mass spectrum indicates that this derivative was derived from an unknown AEP, which in turn was derived from a D-P-D-GlcNAc backbone, and this AEP glycosylates and reduces and reduces.

Figure 1: Fast atom bombardment mass spectrometric analysis of LPSO fractions B and C.

- Panel A: Fast atom bombardment mass spectrum of fraction B.
- Panel B: Fast atom bombardment mass spectrum of fraction C.

FAB-MS analysis of fraction C:
In order to resolve the ambiguities described above, fraction C was permethylated and reanalyzed by FAB-MS to ensure the methylation prior to fragmentation by reverse-phase HPLC. The main carbohydrate-containing peak, fraction C, was analyzed by FAB-MS and gave the spectrum shown in Figure 1B. The resulting mass spectra showed the peaks corresponding to fully permethylated Neu5Ac2GalNAc2PO4 was detected at m/z 1608. An A4-type ion arising from cleavage of the terminal AEP residue was also observed at m/z 1484 corresponding to Neu5Ac2GalNAc2PO4, as well as a series of smaller cleavage ions arising from this ion by successive cleavages. These smaller cleavage ions are observed at m/z 1286 (Neu5Ac2GalNAc2PO4), 1042 (Neu5Ac2GalNAc2PO4), 854 (Neu5Ac2GalNAc2PO4), and 686 (Neu5Ac2GalNAc2PO4). The presence of an ion at m/z 1286, together with the presence of the other ions with lower masses, is crucial in assigning the branching pattern shown in Figure 1B.

Figure 2: Electron impact mass spectrum of a novel derivative, 4-Deoxy1,3-D-ribofuranosyl-1,2,3-trideoxy-1-C-ether. This compound was derived from the methylation linkage analysis of the methylated and reduced fraction A. The ion at m/z 155 probably corresponds to (H2N)2COOCH2(CH2)COO-.
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To investigate the structure(s) of the underlying mucrossack block, a sample of fraction A was dephosphorylated with calf intestinal alkaline phosphatase into a sample of fraction B. This material was subjected to methylation analysis (Table 2), which revealed the loss of terminal Galp, 3-O-substituted Man, and 2,3-di-O-substituted Man and the appearance of terminal Man and extra 2-O-substituted Man, as predicted. In addition, the 4-O-substituted GlcNAc residue and the 6-O-substituted GlcNAc residue were observed, supporting the NMR 3DEXY assignment of the sequence Manα1-4GlcNAcβ1-6Manβ1-4Manβ1-4AHM and Manα1-2Manα1-2Manα1-2Manα1-4AHM standards, respectively (Furman et al., 1991). Fraction E was digested with Aspergillus phosphatase Manα1-3Man-specific α-mannosidase to yield a single compound which eluted at 2.20s. This latter elution position is identical to that of authentic Manα1-4Manα1-4AHM, confirming that the original component terminated in one and two Manα1-2Man α-Mann residues, respectively. Finally, the Fraction E material was digested with jack bean α-mannosidase to produce a compound eluting at 1.50s, which corresponds to free AHM (Furman et al., 1991). Taken together with the linkage analysis of fraction A (which shows only terminal Galp residues) and the 3DEXY data of fraction E (which shows the presence of AHM containing glycans), the presence of a small amount (15%) of Manα1-2Manα1-4Manα1-4AHM suggests that the minor Manα1-3Man containing structure in fraction B is derived from Galβ1-4Manβ1-2Manα1-6Manα1-4K Ericaio1-2Manβ1-4Manβ1-4Manβ1-4AHM.

Figure 5. Disease HPLC analysis and exoglycosidase sequencing of fraction E.
Panel A: Fraction E, unprocessed.
Panel B: Fraction E digested with Manα1-2Man specific α-mannosidase.
Panel C: Fraction E digested with broad specificity jack bean α-mannosidase.
The numbers at the top indicate the elution positions of the glucose oligomer internal standards (Da values).