The Mechanism of Inhibition of Glycosylphosphatidylinositol Anchor Biosynthesis in *Trypanosoma brucei* by Mannosamine*

(Received for publication, May 3, 1993, and in revised form, July 7, 1993)

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In this paper we have analyzed the effects of mannosamine on glycosylphosphatidylinositol anchor and variant surface glycoprotein biosynthesis in the bloodstream form of *T. brucei*. Trypanosomes were biosynthetically labeled with [3H]mannosamine, and [3H]glucosamine in the presence of mannosamine, and the structures of the labeled glycolipids which accumulated were determined. The main glycolipid metabolite of mannosamine was shown to be Mann-Man-GlcN-PI. A trypanosome cell-free system preloaded with this compound was significantly impaired in its ability to synthesize glycosylphosphatidylinositol anchor intermediates beyond Manα1–6Manα1–4GlcNAc1–6PI. This compound is therefore proposed to be an inhibitor of the Dol-P-Man:Manα1–6Manα1–4GlcNAc1–6PI α1→2-mannosyltransferase of the GPI biosynthetic pathway.

In living trypanosomes, 4 mM mannosamine had no effect on protein synthesis but reduced the rate of formation of mature glycosylphosphatidylinositol anchor precursors by 80%. This reduction in anchor precursor synthesis was insufficient to prevent the attachment of glycosylphosphatidylinositol anchors to newly synthesized variant surface glycoprotein molecules. These data suggest that the rate of anchor precursor synthesis in the bloodstream form of *T. brucei*, in contrast to mammalian cells and the procyclic form of *T. brucei*, is in large excess of the cellular requirements for protein anchorage.

Glycosylphosphatidylinositol (GPI)1 membrane anchors are found covalently attached to a wide variety of eukaryotic plasma membrane proteins (Ferguson and Williams, 1988; Low, 1989; Thomas *et al.*, 1990; Cross, 1990; Ferguson, 1991, 1992a). The primary function of GPI anchors is to furnish a stable association between membrane and protein; however, they have also been implicated in a number of other processes including transmembrane signaling in T-cells (Robinson, 1991), a novel endocytic mechanism for the folate-binding protein (Anderson *et al.*, 1992) and apical targetting in polarized cells (Lisanti *et al.*, 1990).

Structural studies (reviewed in Ferguson, 1991, 1992a) of GPI anchors from protozoa, yeast, and mammals indicate a common core structure of ethanolamine-P0-Manα1–2Manα1–6Manα1–4GlcNAc1–6-myo-inositol-1-P04-lipid, where the ethanolamine residue is in amide linkage to the COOH-terminal amino acid α-carboxyl group. The lipid moiety can be a glycerolipid (diacylglycerol, alklyacylglycerol, or lyso-acylglycerol) or, in yeast and slime molds, a ceramide. The conserved core can be variously substituted with carbohydrate and additional ethanolamine phosphate groups. Some GPI anchors contain an additional fatty acid (palmitate) in hydroxy-ester linkage to the inositol ring (Roberts *et al.*, 1988; Ferguson 1992b) which renders them resistant to the action of bacterial phosphatidylinositol-specific phospholipase C (PI-PLC).

The African trypanosome, *Trypanosoma brucei*, is a tsetse fly-transmitted protozoan parasite which divides extracellularly in the mammalian bloodstream, lymph, and interstitial fluids. Each trypanosome expresses a protective surface coat of about 107 copies of GPI-anchored variant surface glycoprotein (VSG). The GPI biosynthetic pathway in bloodstream form *T. brucei* has been recently reviewed (Englund, 1993) and may be summarized as follows: α-GlcNAc is transferred from UDP-GlcNAc to phosphatidylinositol (PI) to form GlcNAc-PI which is de-N-acetylated to GlcN-PI (Doering *et al.*, 1989). Subsequently (Masterson *et al.*, 1989; Menon *et al.*, 1990b), 3 α-Man residues are transferred in single steps, from dolichol-phosphate-mannose (Dol-P-Man) (Menon *et al.*, 1990a), to form Manα1–GlcN-PI. Ethanolamine phosphate is then transferred, from phosphatidylethanolamine (Menon *et al.*, 1993), to the terminal Man residue to form EtN-P-Manα1–

1 The abbreviations used are: GPI, glycosylphosphatidylinositol; GPI-PLD, human serum GPI-specific phospholipase D; JBAM, jack bean α-mannosidase; PI, phosphatidylinositol; PI-PLC, B. *thuringiensis* phosphatidylinositol-specific phospholipase C; PLα2, C. *adama*tateus phospholipase A2; VSG, variant surface glycoprotein; EtN, ethanolamine; sVSG, soluble variant surface glycoprotein; mVSG, membrane variant surface glycoprotein; Dol, dolichol; PAGE, polyacrylamide gel electrophoresis; HPTLC, high performance thin layer chromatography; BSA, bovine serum albumin; TLCK, Nα-p-tosyl-L-lysine chloromethyl ketone; PMSF, phenylmethylsulfonyl fluoride; Gu, glucose unit.
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GlcN-PI (known as glycolipid A'). This species then undergoes a complex series of fatty acid remodeling reactions (Masterson et al., 1990) whereby the two fatty acids of glycolipid A' are sequentially removed and replaced by myristate to yield the true GPI precursor glycolipid A. Concomitant with the formation of glycolipid A is the formation of glycolipid C (the inositol palmitoylated version of glycolipid-specific). The structures of glycolipids A and C (also known as glycolipids P2 and P3) have been rigorously determined (Kraakow et al., 1989; Mayor et al., 1990a, 1990b). Both of these species have been shown to be competent for transfer to VSG polypeptide when added exogenously to a trypanosome cell-free system (Mayor et al., 1991), although there is no evidence of the transfer of glycolipid C (P3) in vivo. The role of glycolipid C is unclear, but it has been suggested to be an obligate intermediate on the pathway to the glycolipid A GPI precursor (Doering et al., 1990; Masterson et al., 1990; Menon et al., 1990b; Menon, 1991). The transfer of GPI precursor to VSG polypeptide occurs in the endoplasmic reticulum and involves the removal of a hydrophobic COOH-terminal GPI signal peptide of 17 or 23 amino acids, depending on the VSG variant (Englund, 1993). The GPI biosynthetic pathway in T. brucei procyclines is similar to bloodstream forms, except that fatty acid remodeling is restricted to the removal of the sn-2-fatty acid and the final precursor contains an inositol palmitate group (Field et al., 1992).

The GPI biosynthetic pathway in mammalian cells is similar to that in trypanosomes, except that all the intermediates are inositol palmitoylated from GlcN-PI onward, and one or two extra ethanolamine phosphate groups are added prior to the completion of the GPI precursor (Hirose et al., 1992; Puoti and Conzelmann, 1992; Kamitani et al., 1992, and references therein). There is no evidence for fatty acid remodeling in mammalian cells. The GPI pathway has also been described recently in the sporozoan parasites Toxoplasma and Plasmodium (Tomavo et al., 1992; Gerold et al., 1992).

The compound 2-deoxy-2-amino-d-mannose (mannosamine) has been shown to be an inhibitor of N-glycosylation (Pan and Elbein, 1985; Pan et al., 1992a) and GPI anchor precursor formation in both mammalian cells and T. brucei procycline forms (Lisanti et al., 1991). Recently, mannosamine has been shown to become incorporated into GPI intermediates in mammalian cells (Pan et al., 1992b) although the exact position(s) of mannosamine incorporation were not determined.

In this paper we have analyzed the structures of all of the major glycolipid intermediates which accumulate in the presence of mannosamine in bloodstream form T. brucei. We conclude that ManN is incorporated almost exclusively into the structure ManMan-GlcN-PI and that this GPI intermediate analogue probably acts as an inhibitor of the Manal-2Man α-mannosyltransferase. We also show that the overall effects of mannosamine on the N-glycosylation of VSG are also discussed.

**EXPERIMENTAL PROCEDURES**

**Materials**—[6-3H]Glcualosamine hydrochloride (40 Ci/mmol), [1,3H]ethanol-1-2-amino hydrochloride (30 Ci/mmol), [9,10-3H]myristic acid (40 Ci/mmol), GDP-(3,4,5-3H)mannose (20 Ci/mmol), and En'Hance liquid and spray were purchased from DuPont NEN, Inc. [6-3H]Mannosamine hydrochloride (20 Ci/mmol) was from American Radiolabeled Chemical, Inc. (supplied by Tocris Neurimin, United Kingdom) and [35S]methionine (>1000 Ci/mmol) was from Amer sham Corp. Aluminum-backed Silica Gel-60 HPTLC plates (Art. 5547) were purchased from Merck (Darmstadt, Germany). Endo-n-acytethylglucosaminidase (Endo-H) and jack bean α-mannosidase (JBAM) were from Boehringer (Mannheim, Germany) and tunicamycin was from Calbiochem. Bacillus thuringiensis PI-PLC was a gift from Dr. MS. Low, Columbia University, New York, New York. Human serum was used as a source of glycosylphosphatidylinositol-specific phospholipase D (GPI-PLD). Crotalus adamanteus phospholipase A2 (PLA2), protease inhibitors, media supplements, mannosamine hydrochloride, UDP-GlcNAc, and Lightening Plus intensifying screens were obtained from Sigma. AG50X12, AG5X4, and Bio-Gel P10 (Bio-Rad) (fresh) were purchased from Bio-Rad and Protein A-Sepharose was from Pharmacia. All other reagents were of analytical grade.

**Metabolic Labeling of Trypanosomes**—Log-phase trypanosomes of the Molteno Institute Trypanozoon antigenic type 1.4 (variant 117) were isolated from the blood of a trypanosome-infected mouse. The cells were resuspended (2 x 10^9/mL) in either glucose-free, glucose- and fatty acid-free, or glucose- and methionine-free medium supplemented with adenosine (12 mg/mL), buctrophorine daffron (10 μg/mL), fatty acid-free BSA (2 mg/mL) bound to myristic acid (4.85 μg/mL), or fatty acid-free BSA alone for fatty acid-free medium, catalase (1 mg/mL), glycerol (35 mM), myo-inositol (20 μg/mL), l-methionine (15 μg/mL), or methionine-free medium, mono-nitroglycerol (56 μM), orornithine (10 μg/mL), sodium pyruvate (1 mM), and sodium HEPES (33 mM), pH 7.4. Trypanosomes were preincubated with the presence of mannose in the absence of mannosamine (0.8 μg/mL) for 40 min at 37°C in a shaking water bath. Radiolabel was then added at 20 μCi/mL (for [3H]glucosamine, [3H]ethanolamine, and [3H]myristic acid) or 100 μCi/mL for [35S]methionine and labeling continued under the same conditions until trypanosomes were harvested by centrifugation for lipid and protein extraction. When labeling with [3H]mannosamine, unlabeled mannosamine was omitted and radiolabel (100 μCi/mL) was added after the trypanosomes had been preincubated in the presence or absence of tunicamycin (0.8 μg/mL) for 20 min as described above. All radiolabels, except for [35S]methionine, were dried and resuspended in the appropriate medium prior to use. Mammalian cell labeling experiments were carried out using [35S]methionine, trichloroacetic acid precipitation of total cell protein from cell extracts indicated that protein synthesis was linear for the duration of the experiment.

**Extraction of Glycolipids**—After metabolic labeling, trypanosomes (3 x 10^8) were centrifuged (20 s, 14,000 x g) and lysed at 1.5 x 10^9/ml in 0.2 M Tris-HCl, pH 8, 2% (w/v) SDS, 1 mM EDTA, 10% (v/v) glycerol, and 5% (v/v) Triton X-100. The lipid extract was dried, dissolved in water-saturated butan-1-ol, and partitioned with water as previously described (Kraakow et al., 1986). The butanol phase was dried in preparation for enzymic or chemical treatments and TLC.

**Extraction, Immuno precipitation, and Endo-H Digestion of Solubiliz eform VSG (sVSG)—Trypanosomes, metabolically labeled with [35S]methionine for 45 min in the presence or absence of mannosamine, were centrifuged (20 s, 14,000 x g) and lysed at 1.5 x 10^9/ml in 0.2 M Tris-HCl, pH 8, 2% (w/v) Nonidet P-40, 5 mM EDTA, 0.1 mM TLCK, and 0.1% (w/v) Triton X-100. The cell suspension was boiled the beads in 0.5% (w/v) SDS, 0.1% (w/v) Triton X-100, 5% (w/v) glycerol, and 5% (w/v) Triton X-100. The cell suspension was boiled at 100°C for 10 min. After cooling to room temperature, beads were removed by centrifugation (15 s, 10,000 x g), and the beads were washed using the buffers described by Owen et al. (1980), except that 10 mg/ml BSA was included in the first two buffers. BSA was released by boiling the beads in 0.5% (w/v) SDS, 0.1 M dithiothreitol for 5 min. After cooling to room temperature, beads were removed by centrifugation and 2 volumes of 0.2 M ammonium acetate, pH 5.5, 2% (v/v) Nonidet P-40, 2 mM PMSF, 0.2 mM EDTA, 0.1 M dithiothreitol were added to the supernatant. The sVSG solution was then incubated in the presence or absence of Endo-H at 250 milliunits/ml for 10 h at 37°C. The digest was terminated by the addition of an equal volume of 2 x concentrated SDS-PAGE sample buffer (3 min, 100°C) and samples analyzed by SDS-PAGE on a 10% resolving gel. Gels were processed for fluorography using En'Hance and exposed against Kodak X-Omat XAR-5 film at -70°C.

**Inhibition of Membrane-Form VSG (mVSG)—Trypanosomes, metabolically labeled with [3H]myristic acid for 45 min in the presence or absence of mannosamine, were centrifuged (10 min, 1300 x g) and
lysed at 5 × 10^6/ml in 10 mM Tris-HCl, pH 7.5, 0.3% (w/v) SDS, 1 mM EDTA, 0.1 mM TLCK, 2 μM leupeptin, 1 mM PMSF at 100 °C for 5 min. After cooling to room temperature, extracts containing mVSG (1 × 10^6 cell equivalents of protein) were diazotized with an equal volume of 2× concentrated SDS-PAGE sample buffer and analyzed by SDS-PAGE as described above.

Chemical Treatments of Radiolabeled Glycolipids—Radiolabeled glycolipids (6000 cpm), dried after partitioning into water-saturated butanol as described above, were resuspended in the appropriate buffer by vortexing and sonication prior to the addition of enzymes. B. thuringiensis PI-PLC digests were performed with 21 μl of 1.6 units/ml enzyme in 20 mM Tris-acetate, pH 7.5, 0.1% (w/v) Triton X-100. GPI-PLD digests were performed in 24 μl of 50 mM Tris-HCl, pH 7.4, 10 mM NaCl, 2.6 mM CaCl₂, 0.1% (w/v) Nonidet P-40 containing 4 μl of whole human serum. C. adamanteus PLA₂ digests were carried out using 5 milliunits/ml enzyme in 40 μl of 25 mM Tris-HCl, pH 7.5, 2 mM CaCl₂, 0.1% (w/v) sodium deoxycholate. JBAM digests were performed using 50 units/ml enzyme in 35 μl of 0.1 M sodium acetate, pH 5, 3.1% (w/v) sodium taurodeoxycholate. All digests were incubated for 16–18 h at 37 °C. In the case of GPI-PLD, PLA₂, and JBAM, enzymes were added in 2 equal aliquots, the second aliquot 4 h after digestion had commenced. After digestion, glycolipids were recovered by extracting twice with water-saturated butanol-1-ol.

The combined butanol phase was back-extracted with water, dried, and resuspended in 10 μl of water-saturated butan-1-ol for TLC analysis.

Chemical Treatments of Radiolabeled Glycolipids—Radiolabeled glycolipids (6000 cpm) were hydrolyzed in 50% (v/v) propan-1-ol containing 1 M HCl for 15 min at 50 °C, for the selective hydrolysis of oligosaccharidyl-PP-dolichol and sugar-PP-dolichol species. Samples were dried, the glycolipids recovered by butan-1-ol partitioning, and prepared for TLC analysis as described above. Samples for N-acetylated glycolipids were recovered by extracting twice with water-saturated butanol-1-ol. The combined butanol phase was back-extracted with water, dried, and resuspended in 10 μl of water-saturated butan-1-ol for TLC analysis.

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RESULTS AND DISCUSSION

In all of the trypanosome labeling experiments, a glucose-free modified Eagle’s medium was used, supplemented with glycerol as carbon source. Trypanosomes were preincubated with 4 mM mannosamine for 40 min before addition of radiolabel. Under these conditions cell viability was good (>95%) over 90 min of labeling. Mannosamine Inhibits the Formation of GPI Precursors in Bloodstream Form Trypanosomes—Trypanosomes were preincubated in the presence and absence of 4 mM mannosamine for 40 min. The cells were then labeled with [3H]ethanolamine or [3H]myristate and glycolipid extracts from a number of time points were analyzed by TLC. The radioactivity associated with glycolipids A and C was measured using a linear analyzer and the kinetics of labeling are shown in Fig. 1.

In the absence of mannosamine, glycolipids A and C label rapidly with [3H]ethanolamine, and approach a steady state after about 25 min of labeling. In the presence of 4 mM mannosamine, glycolipids A and C label slowly with time and the initial rate of labeling is reduced by 80% (Fig. 1a). The incorporation of [3H]ethanolamine into phosphatidyl ethanolamine phospholipid (the ethanolamine phosphate donor species (Menon and Stevens, 1992; Menon et al., 1993)) was unaffected by mannosamine (data not shown), indicating that the inhibition of glycolipid A and C synthesis is due to a direct effect on the GPI biosynthetic pathway.

Similar results were obtained in an identical experiment using [3H]myristate as the biosynthetic label except that the kinetics of glycolipid A and C labeling were slightly different (Fig. 1b). Mannosamine caused a significant inhibition of glycolipid A and C labeling (75%), consistent with an inhibition of GPI biosynthesis by mannosamine. However, both in the presence and absence of mannosamine, the two glycolipids undergo an extremely rapid phase of labeling. This is followed by a slower and more prolonged phase, similar to the labeling seen with [3H]ethanolamine. We attribute this difference in

FIG. 1. The effects of mannosamine on the rates of synthesis of glycolipids A and C. Trypanosomes were preincubated with (+) or without (-) 4 mM mannosamine and subsequently labeled with [3H]ethanolamine (panel a) or [3H]myristate (panel b). Glycolipids were extracted, separated by TLC, and the radioactivity in glycolipids A (circles) and C (squares) was measured using a linear analyzer.

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![Image of glycolipid bands](image)

**Fig. 2. Analysis of [3H]GlcN-labeled glycolipids synthesized in the presence of mannosamine.** Trypanosomes were preincubated with (+) or without (-) mannosamine and tunicamycin as indicated. The extracted glycolipids were treated with (+) or without (-) B. thuringiensis PI-PLC, GPI-PLD, snake venom PLAz, and JBAM as indicated. The glycolipid preparations were analyzed by TLC and fluorography. Control incubations (minus mannosamine) produced predominantly glycolipids A and C, whereas cells preincubated with mannosamine produced predominantly the glycolipid bands X, Y1, Y2, and Z1-4. The sensitivities of these bands to the various treatments shown here are summarized in Table I. To simplify the analysis of the PLA2 sensitivity of band Y1 the formation of Z1 was inhibited with tunicamycin and bands Y2 and Z2-4 were destroyed by treatment with PI-PLC, prior to the PLA2 digestion.

<table>
<thead>
<tr>
<th>Component</th>
<th>Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI-PLC</td>
<td>+</td>
</tr>
<tr>
<td>Tunicamycin</td>
<td>+</td>
</tr>
<tr>
<td>Mannosamine</td>
<td>+</td>
</tr>
</tbody>
</table>

**Table I**

Characterization of the glycolipids that accumulate in the presence of mannosamine

For each glycolipid + indicates its sensitivity to digestion by an enzyme, its destruction by mild acid (H+), or inhibition of its synthesis by tunicamycin (Tn). ND, not determined.

<table>
<thead>
<tr>
<th>Component</th>
<th>Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>+</td>
</tr>
<tr>
<td>Y1</td>
<td>+</td>
</tr>
<tr>
<td>Y2</td>
<td>+</td>
</tr>
<tr>
<td>Z1</td>
<td>+</td>
</tr>
<tr>
<td>Z2</td>
<td>+</td>
</tr>
<tr>
<td>Z3</td>
<td>+</td>
</tr>
<tr>
<td>Z4</td>
<td>+</td>
</tr>
</tbody>
</table>

**Band Y1**—This glycolipid is PI-PLC resistant but GPI-PLD and PLA2 sensitive, which suggests that it has a palmitoylated diacyl-PI phospholipid moiety. The Rf of this band would be consistent with a Man2-GlcN-(palmitoyl)PI (Menon et al., 1990). A small amount (20%) of a 5.0 Gu dephosphorylated, N-acetylated headgroup was identified in the Y fraction (Table II). This would be consistent with an original headgroup of either Man2-GlcN-Ino or ManN-Man-GlcN-Ino. Since the Y bands are well resolved from Man3-GlcN-PI species, which are even slower moving than the Z bands, we suggest that band Y1 has the structure ManN-Man-GlcN-(palmitoyl)PI. This would be the ManN-containing analogue of Man-Man-GlcN-(palmitoyl)PI which is often seen in the cell-free system (Masterson et al., 1989; Menon et al., 1990). One unexpected property of this band is its resistance to jack bean α-mannosidase (Fig. 3, lanes 11 and 12), since terminal non-reducing αMan residues have been shown to be sensitive to this enzyme. However, αManN is a poorer substrate

2 The specificity of jack bean α-mannosidase was investigated using methyl glycosides (ManNa1-O-Me and ManNAcα1-O-Me) as model substrates. ManNa1-O-Me was prepared by methanolysis of ManNAc
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TABLE II

Headgroup analysis of the glycolipids that accumulate in the presence of mannosamine

The [3H]GlcN-labeled headgroup structures shown for regions X, Y, and Z (scraped from a TLC plate similar to that shown in Fig. 2) are assigned according to their size and sensitivity to JBAM. The 5.0 Gu, JBAM-resistant headgroup from peak Z is assigned as containing only one ManNAc residue from its size; in linear structures HexNAc residues generally contribute 2 Gu on Bio-Gel 1987). ND, not determined. Ino, inositol.

<table>
<thead>
<tr>
<th>Starting material</th>
<th>Dephosphorylated, N-acetylated headgroup</th>
<th>Size on Bio-Gel P4 (Gu)</th>
<th>% Minus JBAM</th>
<th>Plus JBAM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standards</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GlcN-P1</td>
<td>GlcNac-Ino</td>
<td>100</td>
<td>2.4</td>
<td>2.4</td>
</tr>
<tr>
<td>Man$_2$-GlcN-P1</td>
<td>Man-Man-GlcNac-Ino</td>
<td>100</td>
<td>4.0</td>
<td>2.4</td>
</tr>
<tr>
<td>Man$_2$-GlcN-P1</td>
<td>Man-Man-Man-GlcNac-Ino</td>
<td>100</td>
<td>4.9</td>
<td>2.4</td>
</tr>
<tr>
<td>(Experimental)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak X</td>
<td>GlcNac-Ino</td>
<td>100</td>
<td>2.4</td>
<td>2.4</td>
</tr>
<tr>
<td>Peak Y</td>
<td>Man-GlcNac-Ino</td>
<td>80</td>
<td>3.0</td>
<td>2.4</td>
</tr>
<tr>
<td>Peak Z</td>
<td>Man-Man-GlcNac-Ino</td>
<td>50</td>
<td>4.0</td>
<td>2.4</td>
</tr>
</tbody>
</table>

Results

Band Y2—Band Y2 is sensitive to PI-PLC and GPI-PLD. It is digested by jack bean α-mannosidase, to form GlcN-P1, and the main headgroup identified in the Y fraction was found to be derived from Man-GlcN-Ino. Taken together these data define band Y2 as Man-GlcN-P1.

Band Z1—This species is a dolichol-phosphate containing species. It is not synthesized in the presence of tunicamycin and it is sensitive to mild acid hydrolysis. As expected it is resistant to both PI-PLC and GPI-PLD. The precise structure of this species is unknown but it does appear to contain ManN because it becomes slightly more apolar upon N-acetylation (data not shown). Given the R$_t$ of this compound we tentatively suggest that it has the structure ManN-GlcNac$_2$-PP-P-Dol. This structure would be consistent with its resistance to jack bean α-mannosidase since the Man-GlcNac bond would be β.

Band Z2—These bands are all sensitive to PI-PLC and GPI-PLD, and to jack bean α-mannosidase which yields GlcN-P1. Non-reducing terminal αMan residues are sensitive to jack bean α-mannosidase so that this latter result does not distinguish GPI intermediates containing αMan in place of αMan per se. In contrast, non-reducing terminal αManNac residues are completely resistant to jack bean α-mannosidase. The headgroup analysis protocol used here involves an N-acetylation step which quantitatively converts ManN and GlcN to their respective N-acetates. Such an analysis of peak Z revealed the presence of headgroups derived from Man-Man-GlcN-Ino and ManN-Man-GlcN-Ino (Table II). These data suggest that Z fraction contains, in addition to the dolichol species Z1, a mixture of Man-Man-GlcN-P1 and ManN-Man-GlcN-P1. The multiple banding pattern seen in the chromatogram is probably due to partial fatty acid remodeling of these species, thus introducing further heterogeneity. Although fatty acid remodeling normally occurs on glycolipid A' (Masterson et al., 1990), it is known to also occur on the Man$_2$-GlcN-P1 species when this is forced to accumulate in the presence of PMSF (Milne et al., 1992).

Presumably Man$_2$-GlcN-P1 species can also remodel when forced to accumulate in the presence of mannosamine.

GPI Species That Label with [3H]Mannosamine—Trypanosomes were labeled with [3H]mannosamine for 90 min, in the presence and absence of tunicamycin, and the glycolipid extracts were analyzed by TLC before and after PI-PLC digestion (Fig. 3, lanes 1–4). The fast moving species, which seems to accumulate in the presence of tunicamycin (Fig. 3, lanes 3 and 4), is most likely Dol-P-ManN. Consistent with this assignment is its sensitivity to mild acid hydrolysis and the similarity of its R$_t$ with authentic trypanosome Dol-P-Man (data not shown). The labeled band seen in the Z region (Fig. 3, lane 1) co-migrates with band Z4. This band is synthesized in the presence of tunicamycin (Fig. 3, lane 3) and is sensitive to PI-PLC (Fig. 3, lanes 2 and 4) and jack bean α-mannosidase (Fig. 3, lanes 8 and 9). Based on these data this band can be defined as [3H]ManN-Man-GlcN-P1. The majority of the label in Z4 is clearly sensitive to jack bean α-mannosidase and is therefore presumably present as

than αMan$^2$ and this factor, combined with the presence of the inositol palmitate group, might be responsible for this effect. In any case, band Y1 is too apolar to postulate the presence of an ethanolamine phosphate group to explain the α-mannosidase resistance. The relatively low yield of the putative ManNAc-Man-GlcNAc-Ino (5.0 Gu) headgroup in the Y fraction may represent poor extraction from the silica from this more hydrophobic inositol-palmitoylated compound.

Fig. 3. Analysis of [3H]ManN-labeled glycolipids. Trypanosomes were labeled with [3H]mannosamine in two separate experiments (lanes 1–4 and lanes 5–8, respectively. The labeling was performed in the presence or absence of tunicamycin as indicated, and the extracted glycolipids were N-acetylated (N-Ac) or treated with PI-PLC or JBAM as indicated. Glycolipid extracts were analyzed by TLC and fluorography. The arrowhead indicates the position of N-acetylated authentic glycolipid C.

(0.5 M HCl in MeOH, 90 °C, 48 h), which produced a mixture of Man$_{N[0]-1}$-O-Me (86%) and Man$_{N[0]-1}$-O-Me (14%) and a trace of ManN (as judged by gas chromatography-mass spectroscopy of their N-[H]acetyl-trimethylsilyl derivatives). Alliquots of this material (about 70 µg) were digested with 30 µl of JBAM (25 units/ml) in 0.1 M sodium acetate, pH 5.0, for 14 h at 37 °C, before and after N-acetylation. Under these conditions (as judged by gas chromatography-mass spectroscopy of the trimethylsilyl derivatives of the N-acetylated products) 30% of the Man$_{N[0]-1}$-O-Me was digested to ManN, and negligible amounts of ManNAc$_{1-2}$-O-Me were digested to ManNAc. Under the same conditions a similar amount of Mana$_{1-2}$-O-Me was completely digested to Man. These data suggest that α-mannosaminides are recognized and cleaved by jack bean α-mannosidase (but at a reduced rate compared with α-mannosides), whereas α-N-acetylmannosaminides are not recognized by jack bean α-mannosidase or are at best extremely poor substrates.

3 W. J. Masterson and M. A. J. Ferguson, unpublished data.
\[ \alpha[^{3}H] \text{ManN}, \alpha[^{3}H] \text{ManN} \text{ to } \alpha[^{3}H] \text{GlcN}, \text{ prior to incorporation, can be inferred from the appearance of a trace of labeled GlcN-PI following jack bean } \alpha-\text{mannosidase digestion (Fig. 3, lane 9).}

A PI-PLC and jack bean \alpha-\text{mannosidase sensitive band, with an } R_{f} \text{ similar to Man}_{2}-\text{GlcN-PI, is seen running between Man}_{2}-\text{Man-} \text{GlcN-PI (Z4) and glycolipid C (Fig. 3, lanes 1, 3, 5, and 8). We tentatively suggest that this may be } \alpha[^{3}H] \text{ManN-Man}_{2}-\text{GlcN-PI, produced by the transfer of } \alpha[^{3}H] \text{ManN to Man}_{2}-\text{Man-} \text{GlcN-PI.}

The presence of labeled species that co-migrate with authentic glycolipids A and C, which are synthesized in the presence of tunicamycin and which show the expected PI-PLC sensitivities (Fig. 3, lanes 1–4) suggests that \alpha[^{3}H] \text{ManN label can be incorporated into glycolipids A and C. However, the } \alpha[^{3}H] \text{ManN-labeled glycolipid C-like band was clearly resolved into two components by } N-\text{acetylation (Fig. 3, lanes 6 and 7). The lower of the two bands in lane 7 co-migrates exactly with an } N-\text{acetylated authentic glycolipid C standard, whereas the upper band is more apolar, suggesting that it may contain an extra } N-\text{acetyl moiety. Since there is no evidence for the incorporation of ManN into the first mannose position and species containing ManN in the second position are incompetent for further elongation, it seems likely that this material has the structure EtN-P-Man}_{2}-\text{Man-Man-GlcN-(palmitoyl)PI. Unfortunately, the extremely low yield of the } \alpha[^{3}H] \text{ManN-labeled glycolipids precluded a more detailed headgroup analysis. The labeling of some apparently authentic glycolipid C with } \alpha[^{3}H] \text{ManN (Fig. 3, lane 7, lower band) can only be explained by conversion of some of the label into some other component of the molecule. The most likely conversion would be epimerization of some of the } \alpha[^{3}H] \text{ManN to } \alpha[^{3}H] \text{GlcN prior to incorporation, as mentioned above. The apparently higher content of epimerized } \alpha[^{3}H] \text{GlcN in glycolipid C, as compared with the earlier intermediate analogues such as Man}_{2}-\text{Man-} \text{GlcN-PI (Z4), may suggest that glycolipid C undergoes significant turnover over 90 min, whereas Z4 does not. Thus, if the epimerization of } \alpha[^{3}H] \text{ManN to } \alpha[^{3}H] \text{GlcN is relatively slow, a greater accumulation of epimerized } \alpha[^{3}H] \text{GlcN in glycolipid C might be expected.}

Taken together the above data strongly suggest that ManN is preferentially incorporated into the second Man position to yield Man}_{2}-\text{Man-} \text{GlcN-PI, but that it can also be incorporated into the third position to form Man}_{2}-\text{Man-Man-GlcN-PI and the corresponding analogues of glycolipids A and C. Since the GPI biosynthetic pathway is unaffected by up to 20 mM mannosamine, using the trypanosome cell-free system (data not shown), it seems likely that the 80% inhibition glycolipid A and C formation seen in vivo is due to the accumulation of the Man}_{2}-\text{Man-GlcN-PI species. This species would be expected to be incapable of acting as an acceptor substrate for the } \text{Man}_{1}-\text{2Man } \alpha-\text{mannosyltransferase, since the 2-OH acceptor site is replaced by an NH}_{2} \text{ group, as discussed by Lisanti et al. (1990) and Pan et al. (1992b). However, it is possible that Man}_{2}-\text{Man-GlcN-PI could play a more active role in the inhibition process. All three Man residues of the GPI anchor are known to be donated directly from Dol-P-Man (Menon et al., 1990), and trypanosomes can incorporate ManN into Dol-P-ManN (Fig. 3, lanes 1–4). The accumulation of substantial amounts of Man-GlcN-PI and Man}_{2}-\text{GlcN-PI in mannosamine-treated cells suggests that the cells are not starved of Dol-P-Man. The fact that this accumulated Man}_{2}-\text{GlcN-PI can proceed only at a greatly reduced rate to glycolipids A and C suggests that the accumulated Man}_{2}-\text{Man-} \text{GlcN-PI may be acting as an } \text{acceptor-analogue inhibitor of the third } \text{Man}_{1}-\text{2Man } \alpha-\text{mannosyltransferase. To test this hypothesis experiments using a cell-free system were performed.}

Man}_{2}-\text{Man-GlcN-PI Inhibits GPI Biosynthesis in a Cell-free System—Trypanosomes were incubated with and without 2 mM mannosamine for 20 min, after which they were harvested, and trypanosome hypotonic lysates were prepared (Masterson et al., 1989). Such lysates constitute cell-free systems which, when charged with UDP-GlcNAc and GDP-[^{3}H]Man, are capable of synthesizing GPI precursors up to and including glycolipid A', the lyso-form of glycolipid A' called glycolipid 8, and glycolipid A", which contains an sn-1-stearoyl-2-myristoyl-glycerol-PI moiety (Masterson et al., 1989, 1990). Triplicate lysate prepared from cells incubated in the absence of mannosamine performed as expected, producing glycolipids A', 8, and A" and some Man}_{2}-\text{GlcN-PI (Fig. 4, lanes 1–3). In contrast triplicate lysates from cells preincubated with mannosamine were significantly impaired in their capacity to synthesize glycolipids A', 8, and A" (Fig. 4, lanes 4–6), whereas they produced comparable amounts of Man}_{2}-\text{GlcN-PI. One interpretation of these results is that the preassembled Man}_{2}-\text{Man-} \text{GlcN-PI present in these membranes acts as an inhibitor of the } \text{Man}_{1}-\text{2Man } \alpha-\text{mannosyltransferase by binding to the enzyme. Clearly the binding is either reversible and/or there is insufficient Man}_{2}-\text{Man-} \text{GlcN-PI to occupy all of the enzyme since reduced but significant (approximately 50%) amounts of glycolipid A', 8, and A" are formed.}

The precise mechanism of inhibition of the Dol-P-Man:Man_{1}-\text{6Man}_{1}-4GlcNAc-1-6PI } \alpha-1-2-\text{mannosyltransferase of the GPI biosynthetic pathway by Man}_{2}-\text{Man-GlcN-PI cannot be deduced from these data. However, Hindsaul et al. (1991) have shown that for a number of glycosyl transfersases there is a critical interaction between the hydroxyl group of the acceptor molecule and an unidentified basic group on the enzyme. This may enhance the nucleophilic character of the acceptor hydroxyl oxygen atom and may lead to its increased rate of reaction with an oxocarboxonium ion derived from the donor, or with a donor-derived glycosyl enzyme intermediate. Thus it is conceivable that the replacement of the acceptor hydroxyl group by an amino group in Man}_{2}-\text{Man-GlcN-PI, which will be protonated under physiological conditions, may give rise to a charge-charge interaction with the basic group on the enzyme (if it is a negatively charged group such as the ionized side chain of Glu or Asp). Such a role for a negatively charged active site base (ionized Glu-148) in the ADP-ribosyltransferase activity of Man}_{2}-\text{Man-GlcN-PI.}

![FIG. 4. The effect of endogenous Man}_{2}-\text{Man-GlcN-PI on in vivo synthesis of GPI intermediates. Trypanosomes were preincubated with and without mannosamine, and cell-free systems (washed membranes) were prepared from these cells. Triplicate aliquots of membranes prepared from control cells (lanes 1–3) and cells preincubated with mannosamine (lanes 4–6) were pulse-labeled with GDP-[^{3}H]Man in the presence of UDP-GlcNAc. Glycolipid extracts were analyzed by TLC and fluorography. The positions of authentic Man}_{2}-\text{GlcN-PI and glycolipid A', 8, and A" standards are indicated by M_{2}, } A', 8, and A" , respectively.](image-url)
diphtheria toxin has previously been discussed by Sinnott (1987). Experiments to address this possible analogy are in progress.

A Reduction in the Rate of GPI Biosynthesis Does Not Affect GPI Anchor Addition to Trypanosome Variant Surface Glycoprotein—The effects of mannosamine on VSG biosynthesis were investigated by precipitating trypanosomes with and without 4 mM mannosamine for 40 min and labeling with [35S]Met. Incorporation of [35S]Met into trichloroacetic acid-precipitable counts was unaffected by ManN (data not shown). However, SDS-PAGE analysis of VSG immunoprecipitates revealed a 57- and 55-kDa doublet of labeled VSG made in the presence of ManN (Fig. 5, lane 3), as compared with a single 57-kDa species in the control (Fig. 5, lane 1). Treatment of the immunoprecipitated VSG with Endo-H produced a single band at 55 kDa in both cases (Fig. 5, lane 1). This result is similar to a previous report (Masterson and Ferguson, 1991), which showed that inhibition of glycolipid A formation by 90% had no effect on the efficient transfer of GPI anchors to VSG. These results suggest that the bloodstream forms of African trypanosomes synthesize GPI precursors at a rate at least 5–10 times higher than that required for GPI attachment to VSG.

Acknowledgments—We thank Anant Menon for making data available prior to publication and Malcolm McConville and T. Rouble for helpful comments and for critical review of the manuscript. We thank Martin Low for the generous gift of PI-PLC.

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