# Biosynthesis of lipophosphoglycan from *Leishmania major*: solubilization and characterization of a ($\beta$1-3)-galactosyltransferase

Ken NG*†‡, Emmanuela HANDMAN* and Antony BACIC†

*The Walter and Eliza Hall Institute of Medical Research, Victoria 3050, Australia and †Plant Cell Biology Research Centre, School of Botany, University of Melbourne, Victoria 3052, Australia

Lipophosphoglycan (LPG) is the major cell surface molecule of promastigotes of all *Leishmania* species. It is comprised of three domains: a conserved glycosylphosphatidylinositol anchor linked to a repeating phosphorylated disaccharide (P2; $PO_4$-6Gal$\beta$1-4Man$\alpha$1-) backbone and capped with a neutral oligosaccharide. In *Leishmania major* the backbone is substituted at the C(O)3 of the Gal*p* residue with side chains containing Gal*p*, Glc*p* and Ara*p* residues whereas in *Leishmania donovani* the backbone is unsubstituted. We report the solubilization of a ($\beta$1-3)galactosyltranferase [($\beta$1-3)GalT] from a *L. major* microsomal preparation using Triton X-100. Solubilization occurs with a 10-fold stimulation of enzyme activity. This ($\beta$1-3)GalT specifically transfers Gal residues from UDP-Gal to exogenously added *L. donovani* LPG acceptor. Depolymerization of the [$^{14}$C]Gal-labelled LPG product with mild acid and analysis by high-performance anion-exchange chromatography detected only the phosphotrisaccharide (P3; $PO_4$-6([$^{14}$C]Gal$\beta$1-3-4Man$\alpha$1-) found in *L. major* LPG. This contrasts with the activity of the membrane-bound enzyme which also synthesizes the larger phosphosaccharide units [Ng, Handman and Bacic (1994) Glycobiology **4**, 845–853]. This suggests that more than one ($\beta$1-3)GalT is involved in the addition of these Gal units and that the solubilized activity is the ($\beta$1-3)GalT that adds the first $\beta$Gal residue to the acceptor. The ($\beta$1-3)GalT was partially purified by lectin-affinity chromatography and used to establish the $K_m$ values for UDP-Gal (445 $\mu$M) and *L. donovani* acceptor (280 $\mu$M as P2 molar equivalent) in kinetic assays. Inhibition studies with various glycosides and mono- and di-saccharides established the P2 repeating unit as the minimum acceptor structure recognized by ($\beta$1-3)GalT. The detergent-solubilized ($\beta$1-3)GalT was reversibly inactivated by millimolar concentrations of univalent anionic salts. The ($\beta$1-3)GalT had an absolute requirement for $Mn^{2+}$ and also required $Mg^{2+}$ for optimum activity; $Mg^{2+}$ cannot substitute for $Mn^{2+}$, which is loosely bound to $\beta$(1-3)GalT and is probably involved in the correct folding of the enzyme. The ($\beta$1-3)GalT was unaffected by $Ca^{2+}$ ions, but were irreversibly inactivated by micromolar levels of transition metal ions ($Cu^{2+} > Zn^{2+} > Ni^{2} > Co^{2+}$). The ($\beta$1-3)GalT activity was also inhibited by diethyl pyrocarbonate, but not by *N*-ethylmaleimide or iodoacetamide, suggesting that active-site histidine residues, rather than cysteine residue(s), are important for enzyme activity.

## INTRODUCTION

Parasites have evolved many specific adaptations that enable them to interact with the host. Such host–parasite interactions are mediated by specialized molecules which hold the key to our understanding of the intricacies of parasitism. The parasitic protozoon, *Leishmania*, is coated by a dense surface layer of glycolipid. Two distinct but structurally related classes of glycolipids have been identified: the lipophosphoglycan (LPG) and the low-molecular-mass glycoinositol phospholipids (GIPLs). The complete primary structure of these molecules has now been established for several species [1].

All LPGs contain a polydisperse phosphoglycan moiety (molecular mass 4–40 kDa) linked to the membrane via a complex glycosylphosphatidylinositol (GPI) anchor which includes a conserved hexasaccharide core moiety. The phosphoglycan is comprised of two domains: a phosphorylated disaccharide repeat unit $PO_4$-6Gal$\beta$1-4Man$\alpha$1- (P2) in the backbone, which can be variably substituted at the C(O)3 position of the Gal*p* residue in a species-specific manner, and a variable terminal non-reducing cap structure containing the Man*p*$\alpha$1-2Man*p*$\alpha$1- structure. The phosphorylated disaccharide repeat units are unsubstituted (*Leishmania donovani*) [2], partially substituted with $\beta$Glc*p* residues (*Leishmania mexicana*) [3] or highly substituted with a complex array of sugars including $\beta$Ara*p*, $\beta$Glc*p* and $\beta$Gal*p* (*Leishmania major*) [4,5].

The LPGs and GIPLs function as protective molecules and act as developmentally regulated virulence factors involved in the recognition and attachment of host macrophages as well as binding to the gut of the sandfly vector [6]. Thus *Leishmania* provides an excellent system for the study of the assembly of glycoconjugates and how changes in the glycoconjugate structure are related to function.

The key to this knowledge will be the isolation, characterization and cloning of the glycosyltransferase enzymes involved. The initial biosynthetic pathways of LPG and protein GPI anchors in *Leishmania* appear to be similar as both molecules contain Man*p*$\alpha$1-4GlcN*p* $\alpha$1-6 linked to a phosphatidylinositol but

Abbreviations used: GalT, galactosyltransferase; GPI, glycosylphosphatidylinositol; GIPLs, glycoinositol phospholipids; LPG, lipophosphoglycan; POR, phosphorylated oligosaccharide repeat; NOR, neutral oligosaccharide repeat; PAD, pulsed amperometric detector; HPAEC, high-performance anion-exchange chromatography; CMC, critical micellar concentration; CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulphonate; DTT, D, L-dithiothreitol; TFA, trifluoroacetic acid; ConA, concanavalin A. The following nomenclature for the PORs and NORs was used: P2, $PO_4$-6Gal$\beta$1-4Man; P3, $PO_4$-6(Gal$\beta$1-3)Gal$\beta$1-4Man; P4a, $PO_4$-6(Ara$\beta$1-2Gal$\beta$1-3)Gal$\beta$1-4Man; P4b, $PO_4$-6(Gal$\beta$1-3Gal$\beta$1-3)Gal$\beta$1-4Man; P5a, $PO_4$-6(Ara$\beta$1-2Gal$\beta$1-3Gal$\beta$1-3)Gal$\beta$1-4Man; P5b, $PO_4$-6(Gal$\beta$1-3Gal$\beta$1-3Gal$\beta$1-3)Gal$\beta$1-4Man; N2, Gal$\beta$1-4Man; N3 (Gal$\beta$1-3)Gal$\beta$1-4Man. All these sugars are in the pyranose configuration.

‡ To whom correspondence should be addressed at: Plant Cell Biology Research Centre, School of Botany, University of Melbourne, Victoria 3052, Australia.

diverge with the addition of the second Man and subsequent sugar residues (Man*p* $\alpha$1-3 in LPG and Man*p* $\alpha$1-6 in protein GPI). Protein GPI anchor biosynthesis is thought to occur on the cytoplasmic face of the endoplasmic reticulum [7], and this is followed by translocation to the lumen and subsequent addition to nascent protein [8]. In LPG biosynthesis the addition of the early core sugars is believed to follow a similar route, but there is evidence to suggest that the subsequent assembly of the phosphoglycan moiety occurs in distinct subcellular compartments within the lumen of the Golgi apparatus [9,10]. Recently a mutant defective in the addition of galactofuranose to the GPI anchor of LPG has been isolated and the genes cloned by functional complementation [11], although whether the gene product is indeed the Gal*f* transferase remains to be elucidated. Two enzymes, ($\beta$1-4)galactosyltransferase and Man$\alpha$1-$PO_4$ transferase, involved in the synthesis of the LPG backbone repeating units, have been characterized in *L. donovani* [12,13] and the activities also identified in *L. major* [14].

We have examined the cell-free membrane-bound ($\beta$1-3)galactosyltransferase [($\beta$1-3)GalT] activities from *L. major* that are involved in adding the branched Gal units to the backbone repeats [14]. These galactosylation reactions can utilize either endogenous *L. major* LPG or exogenously added *L. donovani* LPG, which has an unbranched backbone containing the P2 phosphosaccharide repeating unit ($PO_4$-6Gal$\beta$1-4Man$\alpha$1-) backbone containing the P2 phosphosaccharide repeating unit ($PO_4$-6Gal$\beta$1-4Man$\alpha$1-) backbone, as acceptors. Addition of the branching ($\beta$1-3)-linked Gal units was compatible with a mechanism independent of the synthesis of the backbone. One of the major unresolved questions about the biosynthesis of the branching ($\beta$1-3)Gal units is whether one or more ($\beta$1-3-GalTs are involved. Furthermore purification of the individual enzymes involved would also aid our understanding of the mechanism of the addition of these branched Gal units. In this paper we report the solubilization and partial purification of one of the ($\beta$1-3)GalTs from *L. major* microsomal membranes, and the elucidation of several important properties of its acceptor-binding and catalytic sites.

## MATERIALS AND METHODS

### Materials

The following materials were obtained from Sigma: UDP-Gal, GDP-Man, $\beta$-galactosidase (bovine testes), Gal($\beta$1-4)Man, Hepes, Tris, Mes, TosLys$CH_2$Cl (TLCK), PMSF and leupeptin; UDP-[U-$^{14}$C]Gal (309 $\mu$Ci/$\mu$mol) was from Amersham; Bio-Gel P-10 came from Bio-Rad; alkaline phosphatase (calf intestine) was purchased from Boehringer; Whatman 3 MM paper was from Whatman Paper Co.; octyl-Sepharose CL-4B came from Pharmacia; phosphoinositol-dependent phospholipase C (*Bacillus thuringiensis*) was from Immunotech SA, France. The following detergents were obtained from Boehringer: Triton X-100, Triton X-114, n-octyl glucoside, n-dodecyl maltoside, Mega-10, CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulphonate (CHAPSO), Zwittergent 3-12 and digitonin.

### Parasites

The *L. major* isolate LRC-L137 and the *L. donovani* isolate LRC-L52 were obtained from the WHO Reference Centre for Leishmaniasis in Jerusalem, Israel. The *L. major* virulent cloned line V121 was derived from the LRC-L137 isolate by limit dilution cloning [15] and maintained by passage in Balb/c mice. Promastigotes of both species were grown *in vitro* in Schneider's *Drosophila* medium (Gibco, Glen Waverly, Australia) supplemented with 10 % fetal calf serum.

### Preparation of membranes

Parasites (*L. major* V121 or *L. donovani* LRC-LD52; $10^{11}$ cells) were harvested at late exponential phase, pelleted by centrifugation at 3000 ***g*** for 10 min at 25 °C, washed with PBS, repelleted and suspended in Hepes buffer [100 ml; 50 mM Hepes/NaOH, pH 7.0, 2 mM EDTA (sodium salt), 2 mM EGTA (sodium salt), 50 $\mu$g/ml leupeptin, 1 mM dithiothreitol (DTT), 1 mM TosLys$CH_2$Cl] containing 10 % glycerol. The cells were disrupted by mild sonication at 4 °C (Ultrasonic Disruptor UR200P, Tokyo, Japan) until about 80 % of the cells, as judged microscopically, were ruptured (6 × 10 s bursts at the lowest setting). Large membrane fragments and unbroken cells were removed by centrifugation at 16000 ***g*** for 20 min at 4 °C to produce a clear supernatant. Further ultracentrifugation (105000 ***g*** for 60 min at 4 °C) gave a translucent pellet of crude microsomal membranes. The crude microsomal membranes were resuspended in 10 ml of enzyme buffer (50 mM Hepes/NaOH, pH 7.0, 5 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM DTT, 10 % glycerol, 0.1 mM TosLys$CH_2$Cl and 100 $\mu$g/ml leupeptin) and either used immediately or stored frozen at −70 °C. Most (> 95 %) ($\beta$1-3)GalT activity was recovered in the 105000 ***g*** pellet.

### ($\beta$1-3)GalT solubilization

Crude microsomal membranes from *L. major* were extracted with Triton X-100 at a detergent/protein ratio of 5:1 by weight (1 % Triton X-100, 2 mg/ml membrane protein). Detergent extraction was carried out in an ice-bath (4 °C) with gentle stirring for 1 h. The extract was centrifuged at 105000 ***g*** for 1 h at 4 °C. The 'solubilized membrane proteins' (105000 ***g*** supernatant) were used immediately or stored at −70 °C.

Other detergents (non-ionic detergents, such as digitonin, n-octyl glucoside, n-dodecyl maltoside and Mega-10, and zwitterionic detergents such as CHAPS, CHAPSO and Zwittergent 3-12) were tested at up to 1 to 2 times their critical micellar concentration (CMC), except for digitonin (0.01–0.1 %).

### ($\beta$1-3)GalT assay

The assay of solubilized $\beta$(1-3)GalT activity was a modification of a method previously described for the membrane-bound enzyme [14]. The standard assay contained 10–50 $\mu$g of membrane-bound or solubilized protein in 50 mM Hepes/NaOH buffer, pH 7.0, 5 mM $MgCl_2$, 10 mM $MnCl_2$, 2 % glycerol, 0.1 % Triton X-100, 1 mM ATP, 0.1 mM TosLys$CH_2$Cl, 200 $\mu$M UDP-[$^{14}$C]Gal (0.2 $\mu$Ci; 10 $\mu$Ci/$\mu$mol) and 0.6 mM (measured as molar equivalent) *L. donovani* LPG acceptor in a final volume of 100 $\mu$l. Conditions were varied, and inhibitors added, as indicated in the text. ATP was added to reduce degradation of UDP-gal by contaminating pyrophosphorylase and phosphatase activities. The reaction was allowed to proceed for up to 3 h at 30 °C and terminated by addition of SDS (1 % final concentration). The reaction mixture was spotted on to Whatman 3 MM paper and developed by descending paper chromatography for 20 h using ethanol/1 M ammonium acetate, pH 7 (2:1, v/v) as the developing solvent. The paper was dried, the material remaining at the origin was cut out, and its radioactivity quantified by liquid-scintillation counting using BCS scintillant (Amersham). The counting efficiency for $^{14}$C on paper was 75 % of that obtained in the liquid phase.

### Extraction of LPG

LPG from *L. major* V121 and *L. donovani* LRC-L52 promastigotes in stationary growth phase was obtained by differential solvent extraction as previously described [16,17]. Briefly, GIPLs and lipids were first extracted with chloroform/methanol/water (1:2:0.8, by vol), then LPG was extracted with aq. 9.5% butan-1-ol. The LPG was purified by hydrophobic interaction chromatography using an octyl-Sepharose column equilibrated in 0.1 M ammonium acetate in 5% propan-1-ol and eluted with a gradient of propan-1-ol (5–70%).

### Preparation of LPG fragments

The phosphorylated oligosaccharide repeat units (PORs), P2, P3 [$PO_4$-6(Gal$\beta$1-3)Gal$\beta$1-4Man], P4b [$PO_4$-6(Gal$\beta$1-3Gal$\beta$1-3)-Gal$\beta$1-4Man] and P5b [$PO_4$-6(Gal$\beta$1-3Gal$\beta$1-3Gal$\beta$1-3)Gal$\beta$1-4Man] were obtained by mild acid hydrolysis [40 mM trifluoroacetate (TFA); 8 min; 100 °C) of LPG derived from *L. major* V121. The lipid anchor was removed by passage though a $C_{18}$ cartridge (Walters) and the PORs were purified by adsorption on a DEAE-Sephadex A-25 column and elution with 0.2 M $NH_4HCO_3$. The PORs were than separated by Dionex HPLC as previously described [17]. The corresponding neutral oligosaccharide repeat units (NORs), N3 [(Gal$\beta$1-3)Gal$\beta$1-4Man], N4b [(Gal$\beta$1-3Gal$\beta$1-3)Gal$\beta$1-4Man] and N5b [(Gal$\beta$1-3Gal$\beta$1-3Gal$\beta$1-3)Gal$\beta$1-4Man], were obtained by alkaline phosphatase digestion of the PORs, separated and purified by Dionex HPLC as previously described [17].

### High-performance anion-exchange chromatography (HPAEC)

Desalted NORs and PORs were analysed by anion-exchange chromatography at alkaline pH on a Carbopac PA1 column using a Dionex BioLC carbohydrate analyser (Dionex) equipped with a pulsed amperometric detector (PAD) and an on-line radioactivity monitor (Berthold Instruments). The column was eluted isocratically with solvent A (150 mM NaOH, 25 mM sodium acetate) or solvent B (150 mM NaOH, 187 mM sodium acetate) at a flow rate of 0.6 ml/min. Fractions (0.3 ml) were collected and radioactivity was quantified by liquid-scintillation counting.

### Enzyme digestion

Digestion with alkaline phosphatase (calf intestine; 1 unit) was performed in 100 mM $NH_4HCO_3$, pH 8.0, for 16 h at room temperature; digestion with $\beta$-galactosidase (bovine testes; 0.2 unit) was in 50 mM sodium citrate buffer, pH 5, for 16 h at 37 °C.

### Product characterization

[$^{14}$C]Gal-labelled LPG was produced by incubating Triton X-100-solubilized enzyme from *L. major* V121 with UDP-[$^{14}$C]Gal and *L. donovani* LPG acceptor as described above. After paper chromatography (see above) the material remaining at the origin was recovered by elution with water, and radiolabelled LPG was purified by hydrophobic interaction chromatography (see above). The purified [$^{14}$C]LPG was depolymerized by mild acid hydrolysis using 40 mM TFA at 100 °C for 8 min [4]. The lipid anchor was removed by passage through a $C_{18}$ cartridge (Walters) and the PORs were analysed by HPAEC on a Dionex HPLC system before and after alkaline phosphatase and bovine testes $\beta$-galactosidase digestion (see above).

### Affinity chromatography

A Triton X-100 extract (18 mg of protein) was diluted to 60 ml in concanavalin A (ConA) buffer (50 mM Hepes/NaOH buffer, pH 7.0, containing 5 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM $CaCl_2$, 10% glycerol, 0.1% Triton X-100 and 0.1 mM TosLys$CH_2$Cl). This was loaded on to a column (1 cm × 10 cm) of ConA–agarose pre-equilibrated in ConA buffer. After the column had been washed with ConA buffer (25 ml), the enzyme was eluted with a 120 ml gradient (0–100 mM) of methyl $\alpha$-D-Man*p* in ConA buffer. Fractions (6.5 ml) were assayed for protein and ($\beta$1-3)GalT activity.

### Inhibition of detergent-solubilized ($\beta$1-3)GalT activity by bivalent transition metal ions

Partially purified ($\beta$1-3)GalT (5 $\mu$g of protein) from a ConA–agarose column in 50 mM Hepes/NaOH buffer (pH 7.0) containing 10 mM $MnCl_2$, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 mM TosLys$CH_2$Cl, 10% glycerol and 50 mM methyl $\alpha$-D-Man*p* was assayed for enzyme activity in a final volume of 100 $\mu$l in the presence of additional $CoCl_2$, $NiCl_2$, $ZnCl_2$ or $CuCl_2$. The $I_{50}$ values for $Co^{2+}$, $Ni^{2+}$, $Zn^{2+}$ and $Cu^{2+}$ were determined by secondary plots (double-reciprocal) of the inhibition data obtained with a range of concentration (0–2 mM) of the cations.

## RESULTS AND DISCUSSION

### Effect of detergents on ($\beta$(13)GalT activity

Membrane-bound ($\beta$1-3)GalT can be readily assayed, since products formed on the added LPG acceptor associate with the membranes through the lipid anchor and can be recovered by ultracentrifugation [14]. With detergent-solubilized enzymes, however, an alternative procedure was necessary. This was achieved using descending paper chromatography with ethanol/1 M ammonium acetate pH 7.0 (2:1, v/v) as the developing solvent. During chromatography, proteins and high-molecular-mass polymers, including the LPG acceptor and [$^{14}$C]Gal-labelled LPG product, remain at the origin, while low-molecular-mass material such as UDP-[$^{14}$C]Gal and its hydrolysis product, [$^{14}$C]Gal-1-P, migrate down the paper.

Using this assay we surveyed a range of detergents for their capacity to solubilize ($\beta$1-3)GalT from *L. major* microsomal membranes in an active form. The ($\beta$1-3)GalT activity was compatible with a number of detergents, and most stimulated activity severalfold compared with control incubations without detergent (Table 1). The non-ionic detergents were most effective, with Triton X-100 producing a 10-fold stimulation of activity at its CMC (0.06%). Zwitterionic detergents such as CHAPS, CHAPSO and Zwittergent 3-12 were less effective (2–5-fold stimulation of activity at the CMC). Solubilization of enzyme from membrane vesicles stimulates activity apparently by increasing accessibility of substrate and acceptor. The zwitterionic detergents, however, have a strong inhibitory effect on enzyme activity at concentrations greater than their CMC. In contrast, the enzyme activity was generally more tolerant to non-ionic detergent concentration (Table 1), even though activity was severely inhibited at high concentration (above 5× their CMC; results not shown). Digitonin was the least effective in stimulating ($\beta$1-3)GalT activity but was not inhibitory at high concentration. The mechanism of digitonin solubilization of eukaryotic membranes is through the removal of sterols and is therefore much milder in action than most other detergents [18]. At the lower concentration (0.01%), digitonin probably stimulated activity by permeabilization of membrane vesicles where the enzyme is located on the inside.

**Table 1 Effect of detergents on ($\beta$1-3)GalT activity**

*L. major* microsomal membranes (800 $\mu$g of protein/ml) were assayed for ($\beta$1-3)GalT activity, as described in the Materials and methods section, in the presence of a range of detergents at 1 × CMC or 2 × CMC, except for digitonin. The control incubation contained no detergent. The values in parentheses are fold activation.

| Detergent | Concn. (%, w/v) | ($\beta$1-3)GalT activity (c.p.m./assay) |
|---|---|---|
| None (control) | | 1237 (1.0) |
| Digitonin | 0.01 | 2351 (1.9) |
| | 0.10 | 2687 (2.2) |
| Triton X-100 | 0.06 | 13406 (10.8) |
| | 0.12 | 11026 (8.9) |
| Mega-10 | 0.25 | 9024 (7.3) |
| | 0.50 | 5379 (4.4) |
| n-Dodecyl maltoside | 0.03 | 8729 (7.1) |
| | 0.06 | 10053 (8.1) |
| n-Octyl glucoside | 0.75 | 4163 (3.4) |
| | 1.50 | 1815 (1.5) |
| CHAPS | 0.60 | 4441 (3.6) |
| | 1.20 | 1578 (1.3) |
| CHAPSO | 0.50 | 5610 (4.5) |
| | 1.00 | 3021 (2.4) |
| Zwittergent 3-12 | 0.13 | 2383 (1.9) |
| | 0.26 | 773 (0.6) |

**Table 2 Solubilization of ($\beta$1-3)GalT**

*L. major* microsomal membranes were extracted with Triton X-100 at 4 °C for 1 h at a detergent/protein ratio of 5:1 by weight, in 50 mM Hepes/NaOH (pH 7.0) containing 10% glycerol, 1 mM TosLysCH$_2$Cl, 100 $\mu$g/ml leupeptin and with or without 10 mM MnCl$_2$ and 5 mM MgCl$_2$. MM, microsomal membrane in 1% Triton X-100; SN, solubilized proteins (105000 ***g*** supernatant); P, insoluble membranes (105000 ***g*** pellet) resuspended in enzyme buffer containing 1% detergent. Enzyme activity (diluted 1:10) was assayed in the presence of 10 mM MnCl$_2$ and 5 mM MgCl$_2$ for all fractions.

| Extraction conditions | Total activity (nmol of Gal/h) | Total protein (mg) | Specific activity (nmol of Gal/h per mg) |
|---|---|---|---|
| No extraction (MM) | 139 | 27 | 5 |
| $+Mn^{2+}/+Mg^{2+}$ | | | |
| SN | 170 (86%) | 18 (78%) | 9.5 |
| P | 27 (14%) | 5 (22%) | 5.4 |
| $-Mn^{2+}/-Mg^{2+}$ | | | |
| SN | 14 (8%) | 5.3 (24%) | 2.6 |
| P | 165 (92%) | 16.7 (76%) | 9.9 |

## Solubilization of ($\beta$1-3)GalT

Triton X-100 was chosen for solubilizing ($\beta$1-3)GalT from *L. major* microsomal membranes. Solubilization was defined as activity remaining in the supernatant after centrifugation for 1 h at 105000 ***g***. A 1 h extraction of microsomal membranes at 4 °C with 1% Triton X-100 and 2 mg/ml protein resulted in the solubilization of 86% of ($\beta$1-3)GalT activity and 78% of total protein (Table 2). Bivalent cations ($Mn^{2+}$ and $Mg^{2+}$) are required for effective solubilization, as in their absence only 8% of ($\beta$1-3)GalT activity and 24% of protein were solubilized. The solubilized enzyme was relatively stable in the presence of 10% glycerol and protease inhibitors (TosLysCH$_2$Cl and leupeptin), with retention of 80–85% activity at 4 °C overnight and more than 90% activity at −70 °C over 8 weeks. The enzyme also required the presence of $Mn^{2+}$ for stability on storage.

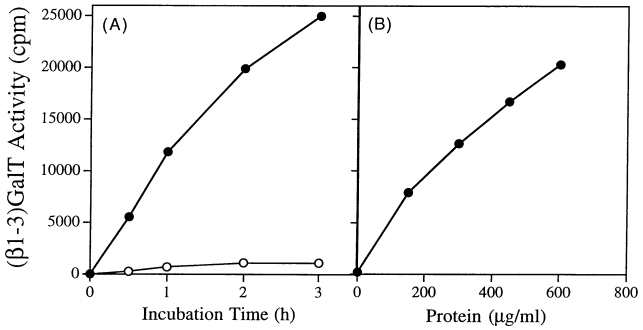


**Figure 1 Effect of incubation time and protein concentration on detergent-solubilized ($\beta$1-3)GalT activity**

(**A**) A Triton X-100 extract containing 60 $\mu$g of microsomal membrane protein was assayed under standard conditions, in the presence (●) or absence (○) of exogenously added *L. donovani* LPG acceptor (see the Materials and methods section), except that the incubation time was varied from 0 to 3 h. (**B**) Protein was varied from 0 to 600 $\mu$g/ml (●) and incubated for 2 h for assay of enzyme activity.

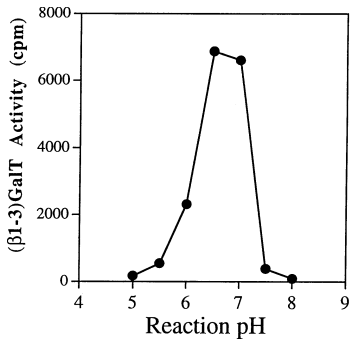


**Figure 2 pH optimum for the detergent-solubilized ($\beta$1-3)GalT activity**

A Triton X-100 extract containing 30 $\mu$g of microsomal membrane protein was incubated at different pHs (5.0–6.5, Mes/NaOH and 7.0–8.5, Hepes/NaOH) under standard conditions.

The detergent-solubilized ($\beta$1-3)GalT activity was constant over time (up to 3 h tested; Figure 1A) and was proportional to the amount of enzyme (up to 600 $\mu$g/ml total protein tested; Figure 1B). In the absence of *L. donovani* LPG acceptor, a low background activity (radioactive material remaining at the origin of the paper chromatogram) of about 5–6% of that assayed in the presence of added LPG acceptor persisted (Figure 1A). This was probably due to the presence of a low level of endogenous LPG acceptor that was solubilized with proteins in the extract, since we have previously shown that these membranes contain endogenous acceptor [14]. However, we have not eliminated the possibility of other endogenous acceptors for the background activity, such as the proteophosphoglycan, which contains P2 repeating units of varying length [19]. A similar molecule is synthesized by *L. major* (Thomas Ilg, personal communication). The galactosylation reaction could also use some endogenous glycoprotein acceptor present in the microsomal membranes. All these products would not be separated in this assay.

The solubilized ($\beta$1-3)GalT exhibited a pH optimum between 6.5 and 7.0 (Figure 2). This contrasts with the broad pH profile reported for the membrane-bound enzyme which retained 75% of maximal activity at pH 5.5 [14], compared with 5% of maximal activity for the solubilized enzyme at this pH. This indicates that the ($\beta$1-3)GalT is more stable at acidic pH values in the membrane-bound form.

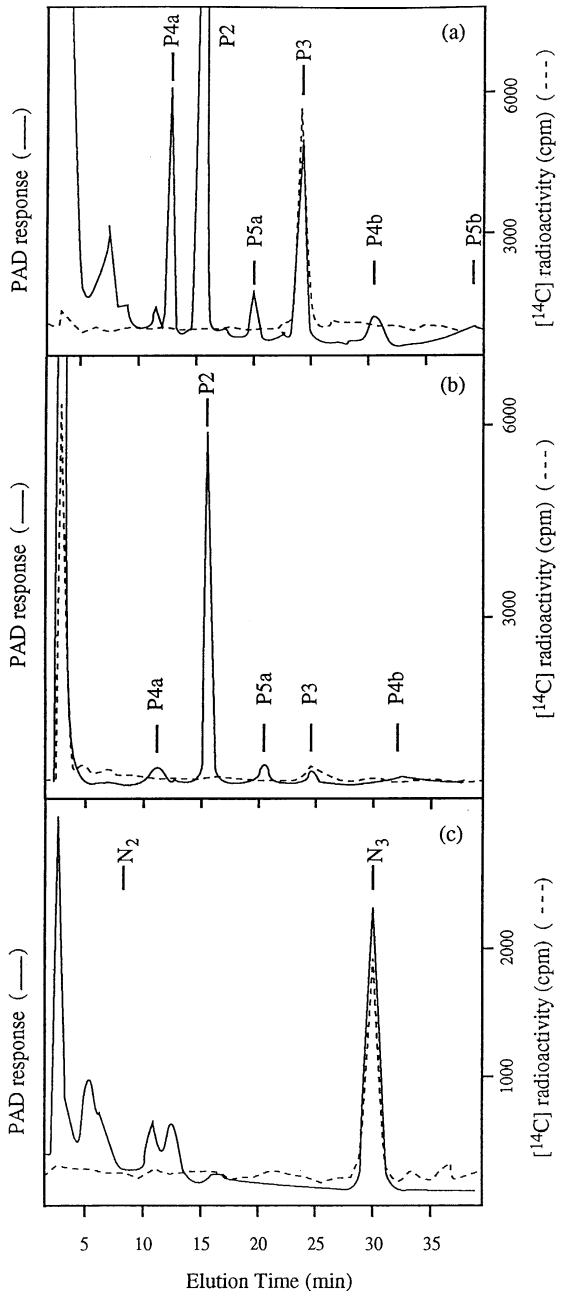


**Figure 3 HPAEC of mild-acid-hydrolysed $^{14}$C-radiolabelled LPG**

[$^{14}$C]Gal-labelled LPG was synthesized by the detergent-solubilized ($\beta$1-3)GalT using *L. donovani* LPG acceptor in a standard assay, purified by paper and hydrophobic interaction chromatography (eluted at 20–30% propan-1-ol from an octyl-Sepharose column), hydrolysed with 40 mM TFA for 8 min at 100 °C and delipidated by passage through a $C_{18}$ cartridge. (**a**) The depolymerized PORs were separated by HPAEC at alkaline pH on a Dionex HPLC system, using a Carbopac PA1 column connected to a PAD and an on-line radioactivity monitor, using solvent A. The major non-radioactive peak material, P2, detected by the PAD was derived from depolymerized *L. donovani* LPG. (**b**) The radioactive peak at 24 min in (**a**) was collected and deionized on Dowex 50 ($H^+$) resin, and a sample digested with bovine testes $\beta$-galactosidase before rechromatography on the Dionex HPLC system using solvent A. The unbound radioactive material is [$^{14}$C]Gal as demonstrated by rechromatography on the Dionex HPLC system using a lower salt (solvent B). (**c**) Another aliquot of the radioactive peak at 24 min was digested with alkaline phosphatase and rechromatographed on the Dinex HPLC system using solvent B. The elution times of standard compounds (P4a, P5a, P3, P4b and P5b) are shown.

Under optimum assay conditions, Triton X-100-solubilized ($\beta$1-3)GalT from $10^{10}$ cells (1 ml packed volume) transferred 0.2 $\mu$mol of Gal/h on to *L. donovani* LPG acceptor at maximum velocity ($V_{max}$). This is equal to $1.2 \times 10^7$ Gal units/h per cell. We know that *L. major* promastigotes contain about $5 \times 10^6$ LPG molecules/cell and each molecule has, on average, 30 P2 repeat units of which 85% are substituted with a single Gal residue [4], and that the turnover rate of cellular LPG is approx. 4 h (Malcolm McConville, personal communication). This would require the addition of about $3.25 \times 10^7$ Gal units/h per cell *in vivo*. The amount of solubilized ($\beta$1-3)GalT activity assayed was thus approx. 2–3-fold less than the estimated *in vivo* rate of LPG biosynthesis in *L. major*.

## Product characterization

To characterize the product(s) of the detergent-solubilized ($\beta$1-3)GalT activity, a scaled-up standard reaction was carried out with the Triton X-100 extract using *L. donovani* LPG as an acceptor and UDP-[$^{14}$C]Gal as the substrate. The [$^{14}$C]Gal-labelled LPG was purified by paper chromatography followed by hydrophobic interaction chromatography on octyl-Sepharose as described in the Materials and methods section. The purified [$^{14}$C]Gal-labelled LPG was depolymerized by mild acid, the lipid anchor was removed by passage through a $C_{18}$ cartridge and the [$^{14}$C]Gal-labelled PORs were analysed by HPAEC on a Dionex HPLC system monitored by pulsed amperometric and radioactivity detection. One radioactive peak (92% of the applied radioactivity) co-eluted with P3 was detected (Figure 3a). Radioactive products co-eluted with larger fragments such as P4b or P5b were not detected. The radioactive P3 peak material was readily digested by bovine testes $\beta$-galactosidase, which has a preference for ($\beta$1-3) linkages, yielding [$^{14}$C]Gal and non-radioactive P2 when rechromatographed on HPAEC (Figure 3b). This is consistent with the removal of a terminal ($\beta$1-3)-linked Gal unit. Cleavage of the orthophosphate group by digestion with alkaline phosphatase, however, resulted in a peak of radioactive material coeluted with N3 (Figure 3c). These data are consistent with the radioactive product having the structure $PO_4$-6([$^{14}$C]Gal$\beta$1-3)Gal$\beta$1-4Man. These results also showed that the detergent-solubilized ($\beta$1-3)GalT, in contrast with the membrane-bound ($\beta$1-3)GalT [14], did not synthesize large oligomers such as P4b and P5b, at least not in amounts that can be detected by the methods described above.

A large number of glycosyltransferases have been studied and they generally follow the one enzyme–one substrate rule [20,21]. An important exception is milk ($\beta$1-4)GalT which transfers Gal from UDP-Gal to a GlcNAc acceptor, but can be induced to utilize UDP-GalNAc with $\alpha$-lactalbumin as substrate [22]. In the case of LPG, branching structures containing two to three ($\beta$1-3)Gal units are common, but large structures with up to 11 ($\beta$1-3)Gal units have been isolated from *L. major* amastigote LPG [5] and from an *L. major* mutant cell line LPG [23]. The acceptor structure for the first galactosylation reaction, which produces branching structures, is a C-3 hydroxy group on a 6-substituted Gal and involves a P2 unit on the *L. donovani* LPG acceptor. The subsequent galactosylation reactions, however, utilize a different acceptor structure, as all are a C-3 hydroxy group on a terminal unsubstituted Gal. On the basis of the different acceptor structures, we can postulate that a minimum of two ($\beta$1-3)GalTs are involved in the synthesis of these branching structures, one to initiate the branch and one to elongate it. This is consistent with the detergent-solubilized $\beta$(1-3)GalT activity that was obtained, which appears to be a single activity responsible for the addition of the first Gal unit on to the *L. donovani* LPG acceptor. There is a possibility that this enzyme is capable of adding more than one Gal unit, but the activity was not

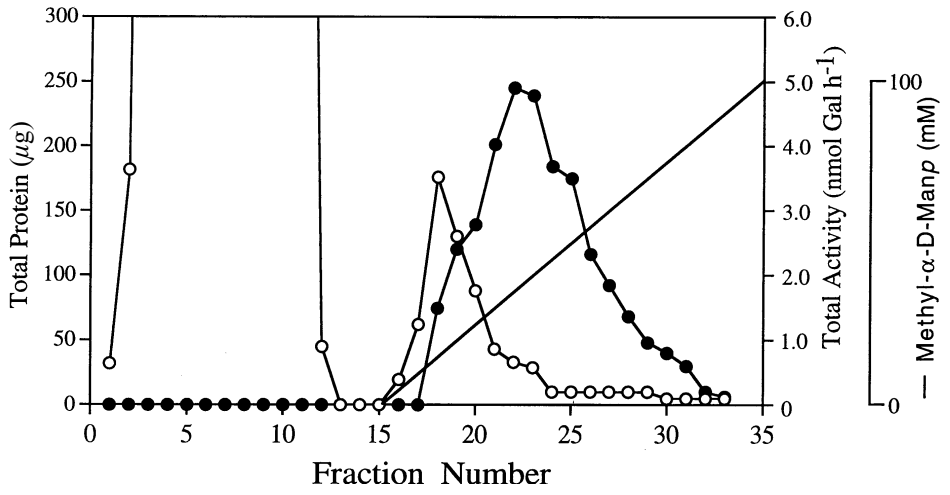


**Figure 4 Affinity chromatography of ($\beta$1-3)GalT on ConA–agarose**

A Triton X-100 extract (18 mg of protein) of *L. major* microsomal membranes was applied to a column (1 cm × 10 cm) of ConA–agarose and, after a washing step, eluted with a gradient (0–100 mM) of methyl $\alpha$-D-Man*p* in ConA buffer. Total protein (○) recovered was 93% (16.7 mg) of which 94.8% (15.9 mg) was not bound and 5.2% (621 $\mu$g) was bound to the column. All the ($\beta$1-3)GalT activity (●) recovered (34% of applied materials) was from the bound fractions. Fractions 20–30 were pooled for further studies (see the legends to Figures 5 and 6 and Table 5).

detected because the concentration of the product of the first transferase reaction (P3) was very low and hence below the $K_m$. This seems rather unlikely since the membrane-bound ($\beta$1-3)GalT readily formed the larger P4b and P5b products using endogenous LPG or exogenously added *L. donovani* LPG acceptors [14]. With *L. donovani* LPG acceptor, a molar ratio of 85:5:1 was obtained for P3/P4b/P5b products synthesized by the membrane-bound ($\beta$1-3)GalT [14]. Furthermore the specific activity of the detergent-solubilized ($\beta$1-3)GalT was 10-fold higher than that of the membrane-bound ($\beta$1-3)GalT (see Table 1). Thus the most likely explanation is that these enzymes exit as a complex or they reside in separate Golgi compartments. In both cases detergent extraction would cause the dissociation of their spatial organization resulting in the loss of the ability to synthesize the larger ($\beta$1-3)Gal side chains.

## Characterization of ($\beta$1-3)GalT

The detergent-solubilized ($\beta$1-3)GalT was not inhibited by either serine peptidase or serine esterase inhibitors, such as PMSF, TosLys$CH_2$Cl, leupeptin and aprotinin. Indeed, the presence of TosLys$CH_2$Cl in the assay gave a higher level of activity, indicating the presence of serine proteases in the detergent extracts that could degrade the enzyme (results not shown). Thiol-alkylating reagents such as *N*-ethylmaleimide and iodoacetamide had little effect on enzyme activity, indicating that free cysteine residues are not involved in binding of substrate or acceptor. The enzyme activity was also stable to prolonged exposure to reducing agents such as DTT (5 mM at 4 °C; 16 h), indicating that disulphide linkages are not necessary to maintain activity.

In order to purify and characterize the enzyme further, a Triton X-100 extract of *L. major* microsomal membranes was chromatographed on a ConA–agarose column. Most proteins did not bind to the column (96% of total recoverable protein; Figure 4). The ($\beta$1-3)GalT activity was bound to the lectin and was eluted at a low to moderate concentration (10–100 mM) of methyl $\alpha$-D-Man*p*, partially resolved from a major peak of bound protein. A substantial amount (66%) of enzyme activity was lost during ConA–agarose chromatography, even though protein recoveries were nearly quantitative (93%). Increasing either methyl $\alpha$-D-Man*p* (to 0.5 M) or detergent (to 0.5% Triton X-100) concentration or increasing the ionic strength using zwitterionic salt (0.5 M betaine, which is compatible with enzyme activity, see below) of the eluting buffer did not improve the recovery of activity from the column, indicating that the loss of activity was not due to some enzyme binding tightly or aggregation of the enzyme on the column (results not shown). Nonetheless, ConA–agarose chromatography readily provides a 10–20-fold enrichment in enzyme activity (100–200 nmol of Gal/h per mg of protein) for kinetic studies. Furthermore the background activity assayed in the absence of added acceptor was substantially less, indicating the removal of contaminant endogenous acceptor(s) during affinity chromatography.

We determined some of the kinetic parameters of ($\beta$1-3)GalT using the partially purified enzyme from ConA–agarose chromatography. The ($\beta$1-3)GalT activity increased in proportion to the amount of added *L. donovani* LPG acceptor up to 0.5 mM P2 molar equivalent before reaching saturation and becoming inhibitory at higher concentrations (Figure 5A). From the secondary plot of the kinetic data, the apparent $K_m$ for the acceptor LPG was 280 $\mu$M, measured as the molar concentration of the repeating unit P2. Assuming an average number of 15 repeating units in *L. donovani* LPG [2], this gave a $K_m$ value of 19 $\mu$M for LPG. The lipid anchor of LPG does not appear to play a part in enzyme recognition and binding; $HNO_2$-deaminated LPG (i.e. *L. donovani* LPG with the inositol lipid anchor removed) was equally effective as an acceptor with a similar $K_m$ (results not shown). This is not surprising since the acceptor domain in the *L. donovani* acceptor, the backbone repeating units, is separated by a hexasaccharide core unit from the inositol lipid moiety.

The ($\beta$1-3)GalT activity follows simple Michaelis–Menten kinetics for the UDP-Gal donor substrate with an apparent $K_m$ of 445 $\mu$M (Figure 5B). UDP showed a classical end-product competitive inhibition of the enzyme activity: increasing UDP

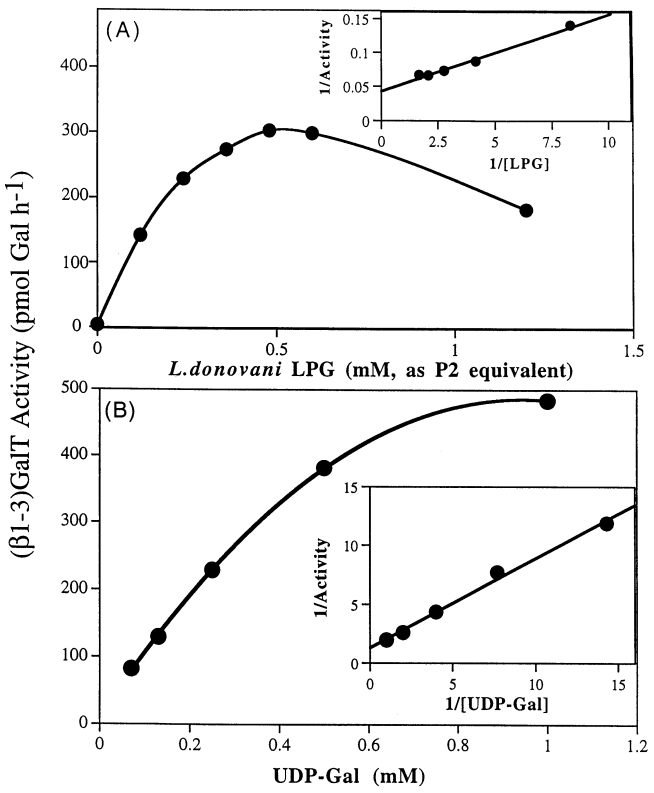


**Figure 5 Effect of *L. donovani* (A) and UDP-Gal (B) concentrations on (β1-3)GalT activity**

A partially purified enzyme (2.5 μg of protein) from ConA–agarose affinity chromatography was assayed for activity under standard conditions but with various amounts of (**A**) *L. donovani* LPG (0–1.5 mM P2 equivalent) acceptor or (**B**) UDP-Gal substrate. Insets show secondary plots (double-reciprocal) of the kinetic data, to obtain $K_m$ values for (**A**) *L. donovani* LPG of 280 μM, measured as the concentration of the repeating unit, P2, or (**B**) UDP-Gal of 445 μM.

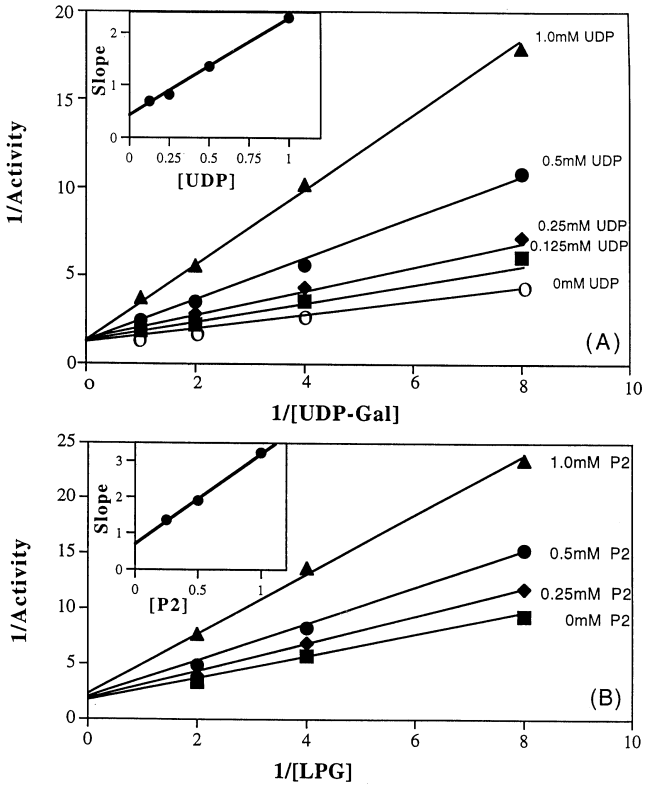


**Figure 6 Inhibition of (β1-3)GalT activity by UDP and P2 phosphodisaccharide**

A partially purified enzyme (5 μg of protein) from ConA–agarose affinity chromatography was assayed for activity. In (**A**) *L. donovani* LPG acceptor was set at a saturating concentration (0.6 mM, P2 molar equivalent) and UDP-Gal concentration was varied from 0.125 to 1 mM with various amounts (0–1.0 mM) of UDP as inhibitor. In (**B**) UDP-gal concentration was set at 200 μM while *L. donovani* LPG acceptor concentration was varied (0.124–0.5 mM, P2 equivalent) in the presence of various concentrations of P2 phosphosaccharide (0.25–1.0 mM) as inhibitor. Insets show (**A**) slope versus [P2] or (**B**) slope versus [UDP] plots of the secondary kinetic data, to obtain a $K_i$ of 280 mM (P2 equivalent) for *L. donovani* LPG and 215 μM for UDP.

concentrations cause an increase in the apparent $K_m$ for UDP-Gal (Figure 6A). From a slope versus [UDP] plot of the kinetic data, a $K_i$ of 215 μM was obtained for UDP.

We examined the acceptor-binding site by using various commercially available sugars and glycosides, as well as the phosphorylated repeat unit, P2, as inhibitors of (β1-3)GalT activity. Neutral sugars such as Man and Gal or glycosides such as methyl α-D-Man*p* and *p*-nitrophenyl β-D-Gal*p* did not inhibit the transfer of Gal from UDP-Gal to the *L. donovani* LPG acceptor. Gal-6-$PO_4$, which resembles the non-reducing terminal of the repeat unit structure, had no significant effect at 0.5 mM, although some inhibition was observed at higher concentration. Galβ1-4Man (N2), the neutral repeating unit structure of LPG, did not inhibit activity up to 10 mM. The phosphorylated $PO_4$-6Galβ1-4Man (P2), however, was a competitive inhibitor with an apparent $K_i$ of 276 μM (Figure 6B). These data indicate that the minimum structural requirements for the binding of the enzyme to LPG acceptor is the phosphorylated repeating unit and that the negatively charged phosphate group plays an essential role. However, we have been unable to demonstrate the transfer of Gal from UDP-[$^{14}$C]Gal to P2 when it is used as the exogenous acceptor. While the phosphorylated disaccharide can occupy the acceptor binding site in competition with *L. donovani* LPG acceptor, the ability of (β1-3)GalT to catalyse the addition of Gal appears to require a more elaborate acceptor structure for correct presentation to the enzyme, possibly involving two or more repeat units in tandem.

The $K_i$ for P2 (276 μM) is in the same range as the $K_m$ for *L. donovani* LPG (280 μM) when expressed as P2 equivalents. This is consistent with more than one enzyme unit binding to a *L. donovani* LPG acceptor molecule. In the Golgi membranes where these enzymes are presumed to be localized, we can envisage the (β1-3)GalTs organized as a cluster where the nascent unbranched LPG molecule is presented as multiple acceptor sites for the branching transferase reactions, similar to a mechanism proposed for the synthesis of heparin sulphate [24]. This would account for the rapid *de novo* synthesis of mature LPG *in vivo* [25,26].

### Effect of salts and metal cations on (β1-3)GalT

We had earlier observed that the membrane-bound (β1-3)GalT was inhibited by high concentrations of univalent salts, such as KCl [14]. In this respect the (β1-3)GalT is similar to a de-*N*-acetylase in GPI anchor biosynthesis [27], and both enzymes differ from the Manα-1-$PO_4$ transferase and the (β1-4)GalT

**Table 3 Effect of univalent salts on detergent-solubilized ($\beta$1-3)GalT**

A Triton X-100 extract was diluted to 0.1% Triton X-100 containing 50 mM Hepes/NaOH, pH 7.0, 10 mM $MnCl_2$, 5 mM $MgCl_2$, 10% glycerol, 0.1 mM $TosLysCH_2Cl$, 420 $\mu$g of protein/ml and with or without 100 mM added salt. ($\beta$1-3)GalT activity was measured using *L. donovani* LPG as acceptor in a standard assay. Values in parentheses are percentages of the control activity.

| Salt | ($\beta$1-3)GalT activity (c.p.m./assay) |
|---|---|
| None | 15643 (100) |
| LiCl | 5435 (35) |
| NaCl | 3637 (23) |
| KCl | 3749 (24) |
| CsCl | 4214 (27) |
| NaF | 10061 (64) |
| NaCl | 3637 (23) |
| NaBr | 1932 (13) |
| NaI | 934 (6) |

**Table 4 Effect of $Mn^{2+}$ and $Mg^{2+}$ on detergent-solubilized ($\beta$1-3)GalT**

A Triton X-100 extract of *L. major* microsomal membranes (30 $\mu$g of protein) containing 10 mM $MnCl_2$ and 10 mM $MgCl_2$ was dialysed against 50 mM Hepes of NaOH (pH 7.0)/0.1% Triton X-100/0.1 mM $TosLysCH_2Cl$/10% glycerol containing (1) $MnCl_2$ (10 mM) and $MgCl_2$ (10 mM), (2) $MnCl_2$ (10 mM) only, (3) $MgCl_2$ (10 mM) only or (4) dialysis buffer alone. The enzyme activity was assayed with or without additional 10 mM $Mn^{2+}$ and/or 10 mM $Mg^{2+}$ cations as shown. Values in parentheses are percentages of the enzyme activity when $Mn^{2+}$ and $Mg^{2+}$ were present in both the dialysis buffer and enzyme assay system.

| | Presence of $Mn^{2+}/Mg^{2+}$ | | |
|---|---|---|---|
| | Dialysis | Enzyme assay | Activity (pmol of Gal/h) |
| 1. | $+Mn^{2+}$, $+Mg^{2+}$ | $+Mn^{2+}$, $+Mg^{2+}$ | 158 (100) |
| 2. | $+Mn^{2+}$ | $+Mn^{2+}$ | 128 (81) |
| | | $+Mn^{2+}$, $+Mg^{2+}$ | 161 (102) |
| 3. | $+Mg^{2+}$ | $+Mg^{2+}$ | 6 (4) |
| | | $+Mn^{2+}$, $+Mg^{2+}$ | 148 (94) |
| 4. | None | None | 0 |
| | | $+Mg^{2+}$ | 5 (3) |
| | | $+Mn^{2+}$, $Mg^{2+}$ | 125 (79) |

involved in LPG backbone biosynthesis [12,13] which are unaffected by salt. We extended this observation on the effect of salts to the detergent-solubilized enzyme, and furthermore found that bivalent cations from the first-row transition elements were potent inhibitors of the enzyme activity.

A typical inhibition experiment using 100 mM univalent salts from the halogen series and the Triton X-100-solubilized ($\beta$1-3)GalT is shown in Table 3. The inhibition of ($\beta$1-3)GalT activity was independent of the cation since LiCl, NaCl, KCl and CsCl had similar levels of inhibition (65–77%), but was dependent on the anion, with $F^- < Cl^- < Br^- < I^-$. Indeed the inhibition correlates with the chaotropic effect of the respective anions in water. This is consistent with the observation that the enzyme activity was compatible with non-chaotropic zwitterionic salts such as taurine (2-aminoethanesulphonic acid), betaine (*N*,*N*,*N*-trimethyl-2-aminoethanoic acid) and sulphobetaine [3-(1-pyridino)butanesulphonic acid] (up to 0.5 M concentration tested; results not shown). Inhibition of ($\beta$1-3)GalT activity by salt is a reversible process. This was shown by recovery of activity by dialysis of a salt-inactivated detergent-solubilized ($\beta$1-3)GalT against buffer lacking salt (results not shown).

The detergent-solubilized ($\beta$1-3)GalT, as for the membrane-bound enzyme [14], has an absolute requirement for $Mn^{2+}$ cations. It also requires $Mg^{2+}$ for optimum activity, but $Mg^{2+}$ cannot substitute for $Mn^{2+}$ in its function (Table 4). Enzymes that require $Mn^{2+}$ for activity fall into two classes, those that contain tightly bound $Mn^{2+}$ and those that are loosely associated with the metal ion [28]. The ($\beta$1-3)GalT belongs to the second class because dialysis of the $Mn^{2+}$-containing enzyme in buffer lacking $Mn^{2+}$ caused inactivation of enzyme activity which could be restored by addition of exogenous $Mn^{2+}$ (Table 4). This implies a role for $Mn^{2+}$ as a cofactor that serves in some structural capacity, rather than being involved in active-site catalysis.

Some of the bivalent cations from the first-row transition elements have a profound inhibitory effect on the detergent-solubilized ($\beta$1-3)GalT activity, with $I_{50}$ in the micromolar range. $Cu^{2+}$ ($I_{50}$ 0.3 $\mu$M) was the most potent, followed by $Zn^{2+}$ ($I_{50}$ 1.8 $\mu$M), $Ni^{2+}$ ($I_{50}$ 293 $\mu$M) and $Co^{2+}$ ($I_{50}$ 812 $\mu$M). Other non-transition-element bivalent cations such as $Ca^{2+}$ (to 5 mM) have no effect, whereas $Mn^{2+}$ (Table 4) was essential for enzyme activity. The inhibition of ($\beta$1-3)GalT activity by the transition metal ions, unlike the univalent salts, was deduced to be specific and irreversible because of the high selectivity and high affinity. Indeed, 80% of a membrane-bound ($\beta$1-3)GalT activity from a microsomal membrane preparation from *L. major* was irreversibly inactivated after 10 min preincubation with 10 $\mu$M $CuCl_2$ and recovery of membranes by centrifugation (results not shown).

The binding site for the inhibitory transition metal ions was expected to be different from that of the $Mn^{2+}$ cofactor because the inhibition of enzyme activity ($I_{50}$) by $Cu^{2+}$, $Zn^{2+}$, $Ni^{2+}$ and $Co^{2+}$ was attained in the presence of a much higher concentration (10 mM) of $Mn^{2+}$. Binding of $Cu^{2+}$, $Zn^{2+}$, $Ni^{2+}$ and $Co^{2+}$ to proteins usually involves aromatic amino acids, in particular histidine, and also cysteine [29,30]. Inhibition of enzyme activity could be accomplished by alteration of protein structure on binding of metal ions or by direct binding to active-site amino acid(s). One example is sucrose synthase [31], in which both surface-accessible and active-site histidine residues are involved in binding to some of the first-row transition elements. Several factors rule out the involvement of free cysteine residues in ($\beta$1-3)GalT activity. The ($\beta$1-3)GalT assay is performed at pH 7, and cysteine, which has a $pK_a$ of 8.4, does not complex efficiently with metal ions because of protonation of the thionate [30]. Furthermore the enzyme activity was insensitive to free thiol-alkylating compounds such as *N*-ethylmaleimide or iodoacetamide.

Histidine is an effective chelator of bivalent metal ions at neutral pH because of the lone pair electron of the nitrogen on the imidazole ring [30]. With a $pK_a$ of 6.1, it is not protonated at pH 7, and can form tight complexes with $Cu^{2+} < Zn^{2+} < Ni^{2+} < Co^{2+}$ [32], which correlates with the inhibitory capacity of these cations on ($\beta$1-3)GalT ($I_{50}$, see above). This strongly suggests that histidine residue(s) of $\beta$(1-3)GalT are involved in metal ion binding. We employed diethyl pyrocarbonate, a reagent that specifically modifies the imidazole ring of histidine residues in proteins [33], as an inhibitor of ($\beta$1-3)GalT activity to probe for the presence of active-side histidine residue(s). A 33% inhibition was apparent at 50 $\mu$M reaching approx. 90% inhibition at 200 $\mu$M (Table 5). Equally significant, the activity could be protected by the presence of an equimolar concentration of histidine in the reaction mixture. This provides evidence that active-site histidine residue(s) are important for ($\beta$1-3)GalT activity.

**Table 5 Effect of diethyl pyrocarbonate on detergent-solubilized ($\beta$1-3)GalT activity**

A partially purified ($\beta$1-3)GalT (2 $\mu$g of protein) from a ConA–agarose column in ConA buffer was assayed for enzyme activity in a final volume of 100 $\mu$l with or without added histidine and/or diethyl pyrocarbonate (DEP). Control reactions contained 5% ethanol because the DEP was introduced as an ethanol solution in the other reaction mixtures. The enzyme activity was unaffected by this concentration of ethanol.

| Conditions | ($\beta$1-3)GalT activity (pmol of Gal/h) | Inhibition (%) |
|---|---|---|
| + 5% EtOH | 146 | 0 |
| 5% EtOH + 200 $\mu$M His | 148 | 0 |
| 5% EtOH + 400 $\mu$M His | 146 | 2 |
| 5% EtOH + 50 $\mu$M DEP | 99 | 33 |
| 5% EtOH + 100 $\mu$M DEP | 53 | 64 |
| 5% EtOH + 200 $\mu$M DEP | 17 | 89 |
| 5% EtOH + 200 $\mu$M DEP + 200 $\mu$M His | 133 | 10 |

The data also indicate that these histidine residue(s) might be involved in binding to the UDP-Gal rather than to *L. donovani* LPG acceptor because the inhibition of activity by the transition element bivalent cations was achieved in the presence of saturating concentration (0.5 mM; P2 equivalents; see Figure 5A) of the acceptor. It remains to be determined whether the histidine residue(s) is involved in catalysis.

## Concluding remarks

The solubilization of active ($\beta$1-3)GalT from microsomal membranes is a prerequisite step to purifying the enzyme. The ($\beta$1-3)GalT is readily solubilized by detergents and the solubilized enzyme is relatively stable. The knowledge gained from studying the acceptor- and substrate-binding sites of ($\beta$1-3)GalT will be invaluable in designing a strategy to purify it, perhaps by using a combination of acceptor or substrate photoreactive analogues in photoaffinity labelling.

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