Leishmania parasites synthesize a range of mannose-containing glycoconjugates thought to be essential for virulence in the mammalian host and sandfly vector. A prerequisite for the synthesis of these molecules is the availability of the activated mannose donor, GDP-Man, the product of the catalysis of mannose-1-phosphate and GTP by GDP-mannose pyrophosphorylase (GDP-MP). In contrast to the lethal phenotype in fungi, the deletion of the gene in Leishmania mexicana did not affect parasite viability but led to a total loss of virulence, making GDP-MP an ideal target for anti-Leishmania drug development. We show by immunofluorescence and subcellular fractionation that GDP-MP is a cytoplasmic protein, and we describe a colorimetric activity assay suitable for the high throughput screening of small molecule inhibitors. We expressed recombinant GDP-MP as a fusion with maltose-binding protein and separated the enzyme from maltose-binding protein by thrombin cleavage, ion-exchange, and size exclusion chromatography. Size exclusion chromatography and analytical ultracentrifugation studies demonstrate that GDP-MP self-associates to form an enzymatically active and stable hexamer. However, sedimentation studies show that the GDP-MP hexamer dissociates to trimers and monomers in a time-dependent manner, at low protein concentrations, at low ionic strength, and at alkaline pH. Circular dichroism spectroscopy reveals that GDP-MP is comprised of mixed α/β structure, similar to its closest related homologue, N-acetylglucosamine-1-phosphate uridylyltransferase (GlmU) from Streptococcus pneumoniae. Our studies provide insight into the structure of a novel target for the development of anti-Leishmania drugs.

The eukaryotic protozoa of the genus Leishmania are the causative agents of several human diseases referred to as leishmaniasis. Symptoms range from self-healing cutaneous lesions to the fatal visceral form of the disease. Leishmaniasis occurs in tropical and subtropical regions of the world, with an estimated 12 million people infected and 350 million at risk of treatment of the disease in regions other than northeast India still relies primarily on the use of pentavalent antimonials, drugs first introduced in the 1930s (2, 3). Moreover, drug resistance has become an increasing problem in the treatment of leishmaniasis (4, 5). It is clear that there is an urgent need for new and specific drugs to combat the disease. With a view to developing such drugs, parasite-specific biochemical and biosynthetic pathways involved in virulence are beginning to be targeted (reviewed in Davis et al. (28)).

Leishmania synthesize a number of unique mannose-containing glycoconjugates that are secreted or that form a thick glycoalyx on the surface of the parasite (6, 7). Several of the membrane-bound glycoconjugates such as lipophosphoglycan (LPG), glycosylinositolphospholipids (GIPLs) (8, 9), and proteophosphoglycan (7, 10) are virulence factors mediating host-parasite interactions in both the sandfly vector and the mammalian macrophage (11).

As is the case in all eukaryotes, Man is a key monosaccharide for the glycosylation of proteins and lipids. The activated donor for the mannosylation of the various mannose-containing biomolecules is GDP-Man. This nucleoside diphosphate sugar is formed by the conversion of mannose-6-phosphate to mannose-1-phosphate by phosphomannomannose isomerase, and its subsequent conversion to GDP-Man by the enzyme GDP-mannose pyrophosphorylase, abbreviated here as GDP-MP (EC 2.7.7.13) (Fig. 1). GDP-Man is transported to the Golgi, where it is incorporated into phosphoglycans, and is also converted to Dol-P-Man, by Dol-P-Man synthase, which is incorporated into N-glycans, glycoinositol phospholipid anchors of proteins and polysaccharides, and in Leishmania, into GIPLs and LPG. GDP-MP is a member of the nucleotidyl transferase family of enzymes. The use of gene deletion mutants lacking enzymes in the Leishmania mannose biosynthetic pathway has indicated that this pathway provides two particularly attractive targets for the development of novel therapeutics. Phosphomannomutase (12) and GDP-MP (13) knock-out parasites were shown to be viable in culture but unable to establish infection in macrophages in vitro or in mice. Here we report the cloning and expression of GDP-MP in Escherichia coli and the characterization of the solution properties of the enzyme. Analytical ultracentrifugation and circular dichroism spectroscopy indicate that the enzyme is a hexamer and has a mix of α/β
structure similar to that of GlmU, an uridylyltransferase from Streptococcus pneumoniae.

**EXPERIMENTAL PROCEDURES**

**Parasite and Bacterial Cultures—Leishmania mexicana promastigotes were grown at 28 °C in M109 medium supplemented with 10% fetal bovine serum. DNA manipulations were performed in *E. coli* strain XL1-Blue (Strategene), and overexpression was performed in BL21(DE3) pLysS cells (Strategene). All *E. coli* strains were cultured in LB medium with the appropriate antibiotics.

Subcloning of the GDP-mannose Pyrophosphorylase Gene Expression—The GDP-MP gene (AJ292039) was amplified by PCR with the primers EcoRI-GFP-F (5’-CCGAAGAATCTGACGATCCGATTGCC-AG-3’) and Sall-GMP-R (5’-ACGGCGCAGCTTACATGTGATCCCGC-CCGT-3’) under the following conditions: denaturation at 94 °C for 1 min followed by 25 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 68 °C for 1 min. The primers were engineered with EcoRI and Sall sites at the 5’ and 3’ ends, respectively, for cloning into the expression vector pMALc2T (New England Biolabs) containing a thrombin cleavage site introduced between the maltose-binding protein (MBP) and GDP-MP.

Expression of Recombinant GDP-mannose Pyrophosphorylase—A 20-ml culture of BL21(DE3)pLysS cells containing the GDP-MP construct incubated at 37 °C overnight was used to inoculate 1.6 liters of LB broth supplemented with 35 μg ml⁻¹ chloramphenicol, 100 μg ml⁻¹ ampicillin, and 0.2% (w/v) glucose. Protein expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.5 mM. The culture was incubated at 37 °C for an additional 2 h before harvesting the cells by centrifugation at 4000 × g for 20 min. The cells were resuspended in 80 ml of column buffer (20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT)) and stored at −20 °C overnight. The cells were thawed and then sonicated on ice in 1-min bursts for a total of 3 min, and the soluble lysate obtained after centrifugation at 9000 × g for 30 min at 4 °C was used to purify the recombinant enzyme.

Purification of Recombinant GDP-mannose Pyrophosphorylase—The MBP-GDP-MP fusion protein was purified from the bacterial lysate by affinity chromatography on amylose resin, according to the manufacturer’s instructions (New England Biolabs). Fractions containing protein were pooled, dialyzed against thrombin hydrolysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 2.5 mM CaCl₂, 0.1 mM DTT), and digested with thrombin for 2 h before harvesting the cells by centrifugation at 4000 × g for 20 min. The culture was incubated at 37 °C for 30 s, and extension at 68 °C for 1 min. The primers were engineered with EcoRI and Sall sites at the 5’ and 3’ ends, respectively, for cloning into the expression vector pMALc2T, a modified version of LB broth supplemented with 35 μg ml⁻¹ chloramphenicol, 100 μg ml⁻¹ ampicillin, and 0.2% (w/v) glucose. Protein expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.5 mM. The culture was incubated at 37 °C for an additional 2 h before harvesting the cells by centrifugation at 4000 × g for 20 min. The cells were resuspended in 80 ml of column buffer (20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT)) and stored at −20 °C overnight. The cells were thawed and then sonicated on ice in 1-min bursts for a total of 3 min, and the soluble lysate obtained after centrifugation at 9000 × g for 30 min at 4 °C was used to purify the recombinant enzyme.

Purification of Recombinant GDP-mannose Pyrophosphorylase—The MBP-GDP-MP fusion protein was purified from the bacterial lysate by affinity chromatography on amylose resin, according to the manufacturer’s instructions (New England Biolabs). Fractions containing protein were pooled, dialyzed against thrombin hydrolysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 2.5 mM CaCl₂, 0.1 mM DTT), and digested with thrombin for 1 h at 37 °C to remove the MBP fusion partner.

The sample was dialyzed overnight at 4 °C against three changes of 100 volumes of 10 mM Tris, pH 7.5, 20 mM NaCl, 2.5 mM DTT prior to purification using ion-exchange chromatography. The protein solution was loaded onto a HiTrap Q Sepharose HP column (Amersham Biosciences). Bound protein was eluted with a linear gradient from 0.02 to 1.0 M NaCl at a flow rate of 1 ml min⁻¹ over 90 min, and eluted fractions were analyzed using SDS-PAGE, Coomassie Blue staining, and immunoblotting with the program SEDFIT (available at www.analyticalultracentrifugation.com). Sedimentation equilibrium data at multiple speeds was fitted to a non-interacting discrete species model of up to three components, self-associating models, or a continuous size distribution model (15) using the program SEDFIT (available at www.analyticalultracentrifugation.com). Sedimentation equilibrium data at multiple speeds was fitted globally to a discrete species model of up to three components or a monomer-trimer-hexamer self-associating model with and without the sedimentation velocity data by employing the program SEDPHAT (also available at www.analyticalultracentrifugation.com). In addition, sedimentation equilibrium data at multiple speeds and protein concentrations of 0.1, 0.3, and 1.0 mg ml⁻¹ were fitted assuming a single species according to Equation 1 as follows.

\[
c(r) = c(r_\text{c})\exp\left(\frac{-r^2}{2\alpha}\right)\]  

(Eq. 1)

where, \(c(r)\) is the concentration at radius \(r\), \(c(r_\text{c})\) is the concentration at the reference radius \(r_\text{c}\), \(\alpha\) is the rotor angular velocity, \(R\) is the gas constant, \(T\) is the temperature, \(M_m\) is the equivalent molar mass assuming a single species, \(\rho\) is the partial specific volume of the solute, \(E\) is the solvent density, and \(E_b\) is the baseline offset.

**Circular Dichroism (CD) Spectroscopy—CD spectra of purified GDP-MP in 20 mM Tris, 0.15 M NaCl, 1 mM DTT, pH 7.5, were recorded on an Aviv 62DS CD spectrophotometer between the wavelengths of 195-250 nm using a slit bandwidth of 1.5 nm. Spectra were collected at a temperature of 20 °C, with a step size of 0.5 nm using a 1-mm path length quartz cuvette. Signal averaging time was 2.0 s, and ellipticities were reported as \(\Delta\Phi\) (deg cm⁻² dmol⁻¹). The concentration of GDP-MP samples employed in the CD studies (0.13-0.88 mg ml⁻¹) were determined by absorption spectroscopy on a Cary 5 UV/Vis spectrophotometer at 280 nm (ε_{280} = 38,370) (16), and CD spectra were fitted using the program CDPRO, which is available at lamol.colostate.edu/~sreeeram/CDPro/.

**Triton X-114 Detergent Phase Separation—**2 × 10⁶ *L. mexicana* parasites washed in phosphate-buffered saline (PBS) were solubilized in 1.0 ml of PBS, pH 7.3, containing 0.5% (w/v) Triton X-114 (TX-114) and 5 μl of protease inhibitor mixture (10 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 80 μM aprotinin, 2 mM leupeptin, 4 mM bestatin, 1.5 mM pepstatin A, 1.4 mM E-64 (Sigma)). The lysate was centrifuged in an Eppendorf centrifuge at 14,000 rpm at 4 °C for 10 min to remove insoluble material, and the proteins were fractionated into a hydro-
phic water fraction and a hydrophobic detergent fraction, as described (17).

**Immunoprecipitation of Native GDP-MP**—Protein G-Sepharose beads (Amer sham Biosciences) were washed twice with TBS-Triton (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Triton X-100) and dispensed into 20-μl aliquots in microcentrifuge tubes. 30 μl of either normal rabbit serum or rabbit antiserum raised against recombinant GDP-MP was added to 20 μl of beads and incubated on ice for 2 h. L. mexicana lysate was prepared by solubilizing parasites in TBS-Triton. Insoluble material was removed by centrifugation at 18,000 × g for 10 min. 120 μl of parasite lysate, representing 1.5 × 10⁷ parasites, was added to 20 μl of antibody-coated beads and incubated on ice for 2 h. The beads were washed three times in TBS prior to the bound enzyme being either assayed for activity or analyzed by Western blotting. Mouse antibodies against recombinant GDP-MP were used to detect duplicate samples by Western blot analysis.

**Western Blot Analysis**—Parasite and bacterial lysates, as well as enzyme-containing fractions, were analyzed by SDS-PAGE on 10% acrylamide gels, and immunoblot analysis was performed as described (18) using rabbit antibodies to histidine-tagged GDP-MP followed by horseradish peroxidase-conjugated sheep anti-rabbit or anti-mouse IgG (Chemicon). Antibody binding was detected by chemiluminescence following the manufacturers’ instructions (ECL kit, Amersham Biosciences).

**Immunofluorescence Analysis**—L. mexicana promastigotes in PBS were allowed to settle onto poly-L-lysine coated slides for 2 min and fixed with 1% (v/v) formaldehyde, 2% (v/v) glucose in PBS. After permeabilization with 0.05% saponin in 1% fetal bovine serum in PBS, GDP-MP was detected with rabbit antibodies raised to the recombinant protein followed by fluorescein isothiocyanate-conjugated anti-rabbit Ig secondary antibody (Silenus).

**GDP-mannose Pyrophosphorylase Activity Assay**—Enzyme activity was determined using a colorimetric assay coupled with inorganic pyrophosphatase (19). The assay was carried out at 30 °C for 30 min in a 100-μl reaction containing 50 mM Tris (pH 7.5), 8 mM MgCl₂, 100 μM GTP, 100 μM mannose 1-phosphate, 1 mM DTT, 0.1 units ml⁻¹ inorganic pyrophosphatase (Sigma), and 0.1 μg of recombinant GDP-MP. For detection, 100 μl of 0.03% (w/v) malachite green, 0.2% (w/v) ammonium molybdate, and 0.05% (w/v) Triton X-100 in HCl were added, and the reaction was incubated for a further 5 min at 30 °C. The inorganic pyrophosphate generated in the reaction is immediately converted to inorganic phosphate by an excess of inorganic pyrophosphatase. The increase in the concentration of inorganic phosphate, representing GDP-MP activity, was determined by measuring the absorbance at 650 nm. Activity was expressed as change in OD₆₅₀/0.1 μg of enzyme/30 min.

**RESULTS**

**Expression and Purification of Recombinant L. mexicana GDP-mannose Pyrophosphorylase**—The L. mexicana gene encoding GDP-MP was subcloned into the expression vector pMALc2T, containing an N-terminal MBP tag, and expressed in E. coli. The recombinant fusion protein was initially purified on amylose resin, and the MBP tag was subsequently removed by hydrolysis with thrombin. GDP-MP was separated from the MBP by ion-exchange chromatography followed by size exclusion chromatography (Fig. 2A). The purification resulted in a single protein band with the relative molecular mass of 41.9 kDa, equivalent to a monomer when electrophoresed in the absence of reducing agent (Fig. 2A). This was confirmed by Western blotting using antibodies to GDP-MP, which recognized both the recombinant fusion protein and the purified enzyme (Fig. 2B). In contrast, antibodies to the MBP tag recognized the fusion protein but not the purified enzyme (Fig. 2B).

**Enzymatic Activity of Recombinant GDP-MP**—The enzymatic activity of recombinant GDP-MP was determined in a colorimetric assay coupled to inorganic pyrophosphatase. The activity of the recombinant enzyme was assayed prior to, and following, the removal of MBP by thrombin hydrolysis and ion-exchange chromatography. The enzyme activity of chimeric GDP-MP fused to MBP was determined to be 1.762
OD$_{650}$/0.1 μg of enzyme/30 min. The pyrophosphorylase activity following the removal of the MBP moiety and further purification of the GDP-MP by size exclusion chromatography (see below) was calculated to be 1.842 OD$_{650}$/0.1 μg of enzyme/30 min, very similar to that of the fusion protein. The activity of the recombinant enzyme was similar to that obtained for the native enzyme immunoprecipitated from parasite lysates (Fig. 3).

**Cellular localization of GDP-MP**—Analysis of the amino acid sequence of the enzyme suggested that the protein is water-soluble. We set out to confirm the solubility properties of GDP-MP and determine the cellular localization of the enzyme. We examined the presence of the enzyme in wild type *L. mexicana* promastigotes and as a specificity control in the null mutants ΔGDP-MP. Western blotting analysis of the proteins in the Triton X-114 detergent and aqueous fractions after detergent phase separation showed that the enzyme is present in the water phase (Fig. 4A). No protein was detected in the null ΔGDP-MP parasites or in the Triton X-114 phase of the wild type parasites. The cytoplasmic localization of the protein was confirmed by immunofluorescence, with the anti-GDP-MP antibodies showing that the protein was distributed throughout the parasite cytosol, with no staining in any organelle or on any membranes (Fig. 4B). As expected, no signal was detected in the ΔGDP-MP parasites stained with the same antibodies.

**Characterization of GDP-MP by Analytical Size Exclusion Chromatography and Sedimentation Velocity**—When subjected to analytical size exclusion chromatography in the presence of reducing agent, GDP-MP elutes as a major peak with a relative mass of 207 kDa calculated from a four-point standard curve (Fig. 5). This observation suggests that GDP-MP exists primarily as a non-covalently associated pentamer or hexamer in aqueous solution. Sedimentation velocity analysis of the peak fraction after size exclusion chromatography (Fig. 5, t~52 min) confirmed that GDP-MP exists as a hexamer, with a molar mass calculated from the ordinate maximum of the c(M) distribution of 246 kDa (Fig. 6B). This is in good agreement with the theoretical molar mass for the hexameric form of GDP-MP (Table I). In addition, the data shown in Fig. 6A were also fitted to a continuous sedimentation coefficient, c(s), distribution (15). Based on the c(s) distribution best-fit (Fig. 7A, solid line), the hexamer has a sedimentation coefficient of 9.5 S with an axial ratio assuming an ellipsoidal structure of ~5.2, indicating that this oligomer is asymmetric in structure (Table I). However, Fig. 5 shows that the sample also contains higher molar mass aggregates, as well as material corresponding in size to monomers and trimers. Accordingly, the quaternary structure of GDP-MP was further characterized by analytical ultracentrifugation in varying solution conditions.
**TABLE I**

Hydrodynamic properties of recombinant GDP-MP

| Oligomer    | $M_r$ $^a$ | $s_{20,w}$ $^b$ | $\gamma_c$ $^c$ |
|-------------|------------|-----------------|----------------|
| Monomer     | 41.9       | 2.5             | 8.0            |
| Trimer      | 126        | 5.7             | 6.1            |
| Hexamer     | 251        | 9.5             | 5.2            |

$^a$ Molar mass based on amino acid sequence.
$^b$ Taken from the ordinate maximum of the corresponding peak in the continuous-size distribution best-fit (Fig. 6A) (15).
$^c$ Axial ratio ($\gamma_c$) calculated using the $\iota$ method (14).

**Fig. 7.** Continuous size distribution analyses of GDP-MP in varying solution conditions. The continuous sedimentation coefficient distribution, $c(s)$, is plotted as a function of the standardized sedimentation coefficient, $s_{20,w}$ (Svedberg), for GDP-MP at an initial protein concentration of 0.3 mg/ml. A, $c(s)$ distribution of GDP-MP solubilized in 20 mM Tris, 0.15 M NaCl, 1 mM DTT, pH 7.5, at $t = 0$ (solid line) and $t = 5$ days (dashed line). B, $c(s)$ distribution of GDP-MP dissolved in 20 mM Tris, 1 mM DTT, pH 7.5 (dash-dot line), and 20 mM Tris, 1 M NaCl, 1 M DTT, pH 7.5 (solid line). C, $c(s)$ distribution of GDP-MP solubilized in 20 mM Tris, 0.15 M NaCl, 1 mM DTT, pH 7.5 (solid line) and 20 mM glycine, 0.15 M NaCl, 1 mM DTT, pH 10.0 (dash-dot line). Continuous size distributions were calculated at a resolution of 200 sedimentation coefficients between 0.5 and 12.0 S, at $p = 0.95$ and $f/f_o = 1.45$ using the program SEDFIT (15). The r.m.s.d. for all fits was $\leq 0.01$.

**TABLE II**

Concentration-dependent self-association of GDP-MP

| Protein concentration | $M_{eq}$ $^a$ | r.m.s.d.$^b$ |
|-----------------------|---------------|-------------|
| 0.1                   | 222 ± 0.34    | 0.0041      |
| 0.3                   | 233 ± 0.29    | 0.0061      |
| 1.0                   | 236 ± 1.21    | 0.0071      |

$^a$ Equivalent molar mass ($M_{eq}$) calculated from Equation 1.
$^b$ r.m.s.d. for nonlinear global best-fit to sedimentation equilibrium data at 10,000 and 18,000 rpm according to Equation 1.

**DISCUSSION**

Until the deletion of the GDP-MP gene in *L. mexicana* (13), it was generally assumed that the biosynthesis of mannose-containing glycoconjugates was of vital importance to all eukaryotic organisms (20). Deletion of the gene in *Candida* and *Saccharomyces cerevisiae* is lethal (21), and no natural mutants have been observed in mammals including humans. Mannose is essential for the glycosylation of proteins and lipids, and the resultant glycoconjugates, such as protein N- and some O-glycans, glycosylphosphatidylinositol protein membrane anchors, and some glycolipids, have a variety of important functions. In *L. mexicana*, GDP-MP was shown to be essential for the formation of GDP-Man (13), the activated mannose donor for all mannosylation reactions in *Leishmania spp*. These parasites lacked all mannose-containing glycoconjugates.

$^2$ J. Vistica, J. Dan, A. Balbo, E. Yikilmaz, R. A. Marivzza, T. A. Rouault, and P. Schuck, submitted.
coconjugates such as LPG, proteophosphoglycans, glyco-
sylphosphatidylinositol-anchored proteins, GPIs, and N-glycans. Also lost was the newly discovered β1-2 mannan, considered important in protection from stress. It has been suggested that the failure of the GDP-MP mutants to accumulate β1-2 mannan may account to a large extent for their lack of survival in vivo (22). The ΔGDP-MP parasites were viable in cell-free culture but were unable to survive in macrophages or mice, making it a very attractive target for anti-Leishmania drug design.

In this study, the entire Leishmania GDP-MP gene was cloned and expressed as a recombinant protein. The gene sequence and preliminary studies (13) suggested that the protein was water-soluble and most likely cytoplasmic. We used subcellular fractionation with Triton X-114 (17), which showed that the enzyme partitioned into the water phase. Immunofluorescence confirmed the cytoplasmic localization of the enzyme. Furthermore, using an adaptation of a coupled colorimetric assay described for Candida, we show that the recombinant enzyme has enzymatic activity both as a fusion protein with the E. coli maltose-binding protein and after hydrolysis by thrombin. The specific activity is similar to that observed for the native protein and for the homologous enzyme in Candida albicans.

Size exclusion chromatography and analytical ultracentrifugation experiments demonstrate that GDP-MP self-associates to form a hexamer in the presence of reducing agent (DTT) and is stable under physiological conditions. The early study of the GDP-MP deletion mutants showed that the enzyme activity in the parasite lysate was detected in the size range of 240–300 kDa, supporting the idea that the enzyme is a hexamer. Although the hexamer is shown to dissociate to trimers and monomers slowly over time, in low ionic strength, and particularly, at alkaline pH, the hexamerization of GDP-MP is stabilized at high protein concentrations (Table II) more similar to the crowded intracellular environment in the parasite (23). Hexamerization was also stabilized with the addition of high salt (1.0 M). These observations suggest that the self-association of GDP-MP is driven by electrostatic, hydrogen-bonding, and hydrophobic interactions and not dominated by any one type. Furthermore, based on sedimentation velocity experiments conducted at low ionic strength, GDP-MP is likely to self-associate from asymmetric monomers (a/b 5.2) to more symmetrical higher order oligomers, namely trimer (a/b 6.1) and hexamer (a/b 5.2) (Table I).

A search of the domain data base, Pfam (www.sanger.ac.uk/Software/Pfam), indicates that in addition to the nucleotidyl transferase domain at the N terminus, GDP-MP contains four hexapeptide domains in the C-terminal region of the protein. Tandem repeats of hexapeptide domains have been shown to form a left-handed β helix in bacterial transferases (24). By inference, it may be postulated that GDP-MP may also exhibit left-handed β helix structure in the C-terminal domain of the protein.

Using the fugue server (www.cryst.bioc.cam.ac.uk/~fugue/) to find structural homologues of GDP-MP, Z scores in excess of 30 were found for several related UDP-N-acetylglucosamine pyrophosphorylases. The preferred alignment of GDP-MP was with the pyrophosphorylase domain of the bi-functional bacterial enzyme N-acetyl-glucosamine-1-phosphate uridyltransferase (Glmu) from S. pneumoniae (25) and E. coli (26). The Glmu molecule, which self-associates as a trimer, consists of a globular pyrophosphorylase domain and a left-handed β helix (LβH) domain connected by an α-helical linker. The trimeric subunit interface of Glmu is formed by three copies of the LβH domain and is thought to contribute to the stabilization of the molecule, in addition to forming the acetyl CoA binding site (25, 26). In eukaryotes, there is no bifunctional equivalent of Glmu, and the activities of the acetyltransferase and pyrophosphorylase domains are accomplished by two distinct enzymes (25). In GDP-MP, the LβH...
domain may therefore play a role in the stabilization of the quaternary structure of the enzyme without conferring an additional enzymatic activity upon the molecule. In the crystal structure of Glmu from E. coli (26), the C-terminal domains were reported to pack base-to-base to form a hexameric aggregate.

Based on these considerations, and on our data showing that the GDP-MP hexamer dissociates to trimers at low ionic concentration, one can propose that the GDP-MP hexamer is composed of a dimer of trimers driven by non-covalent interactions. The Leishmania Golgi GDP-Man transporter, LPG2, which transports GDP-Man from the cytoplasm to the lumen of the Golgi, has also been shown to form hexameric complexes (27), indicating that hexamerization occurs in more than one protein involved in glycosylation in these organisms.

Since Leishmania parasites lacking the enzyme are totally avirulent, inhibition of this enzyme activity provides an attractive target for anti-Leishmania drug design. Given that the stability of the GDP-MP hexamer is compromised at low ionic strength and at high pH, we propose that new drug candidates can be designed to inhibit the active hexameric form of this enzyme. The availability of the recombinant protein and the enzyme assay described here, which is suitable for high throughput screening for small molecule inhibitors, should facilitate the drug discovery process.

Acknowledgments—We thank Geoff Howlett and Peter Colman for input throughout this study and for critically reviewing this manuscript, Peter Schuck for providing technical support for the analysis of sedimentation data with SEDPHAT, and Jim Goding for providing the modified pMalc2T vector, which made the production of the recombinant protein possible.

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