The final two steps of de novo uridine 5’-monophosphate (UMP) biosynthesis are catalyzed by orotate phosphoribosyltransferase (OPRT) and orotidine 5’-monophosphate decarboxylase (OMPDC). In most prokaryotes and simple eukaryotes these two enzymes are encoded by separate genes, whereas in mammals they are expressed as a bifunctional gene product called UMP synthase (UMPS), with OPRT at the N terminus and OMPDC at the C terminus. Leishmania and some closely related organisms also express a bifunctional enzyme for these two steps, but the domain order is reversed relative to mammalian UMPS. In this work we demonstrate that L. donovani UMPS (LdUMPS) is an essential enzyme in promastigotes and that it is sequestered in the parasite glycosome. We also present the crystal structure of the LdUMPS in complex with its product, UMP. This structure reveals an unusual tetramer with two head to head and two tail to tail interactions, resulting in two dimeric OMPDC and two dimeric OPRT functional domains. In addition, we provide structural and biochemical evidence that oligomerization of LdUMPS is controlled by product binding at the OPRT active site. We propose a model for the assembly of the catalytically relevant LdUMPS tetramer and discuss the implications for the structure of mammalian UMPS.

Pyrimidine nucleotides are essential for all forms of life not only as RNA and DNA precursors, but also in signaling, cell membrane assembly, and phospholipid, complex lipid, and glycoconjugate biosynthesis (1–3). The synthesis of pyrimidine nucleotides in nature can occur de novo from small molecule metabolites and by salvage of preformed pyrimidine bases. Some microorganisms, such as Giardia lamblia and Entamoeba histolytica (4), lack the de novo pathway and rely exclusively on pyrimidine salvage for their pyrimidine needs. For higher organisms, the relative contribution of the de novo and salvage pathways is dependent upon cell type and stage of development. The de novo pathway is important in proliferating cells and, as such, is up-regulated in tumors and neoplastic cells (5). As a result of differences between organisms and cell types, enzymes involved in pyrimidine biosynthesis and salvage are important targets for antimicrobial and anticancer agents. In addition, the importance of these pathways has been linked to the etiology or treatment of other diseases including diabetes, AIDS, and rheumatoid arthritis (6–8).

The de novo pyrimidine biosynthetic pathway involves six enzymatic steps leading to the synthesis of uridine 5’-monophosphate (UMP) (Fig. 1A). The final two enzymes in UMP biosynthesis, orotate phosphoribosyltransferase (OPRT) (9) and orotidine 5’-monophosphate decarboxylase (OMPDC), catalyze the synthesis of orotidine 5’-monophosphate (OMP) from orotate and 5-phosphoribosyl 1-pyrophosphate (PRPP), and the decarboxylation of OMP, respectively, leading to UMP. OMPDC is a cofactor-independent decarboxylase that is known to be one of the most kinetically efficient enzymes yet discovered, providing a 10^{17} rate enhancement over the non-enzymatic reaction (k_{cat}/k_{non}) (9). In the course of studying its remarkable catalytic ability, many structures of this enzyme have been determined since the first structures were reported in 2000 (10–13). These include several structures of the OMPDC domain of human UMP synthase (HsUMPS), a bifunctional protein consisting of both OPRT and OMPDC domains (14, 15). OPRT is a transferase important for cancer chemotherapy as an activator of the prodrug 5-fluouracil (16). There are also several available structures of OPRT (17–19) including a recent structure of the human OPRT domain of UMP synthase (PDB code 2WNS).

Although the chemistry in this pathway is largely conserved throughout all kingdoms of life, the organization of the genes varies. The six steps are catalyzed by six separate gene products in most prokaryotes and five in lower eukaryotes, whereas mammalian UMP biosynthesis employs a trifunctional enzyme for the first three steps and bifunctional UMPS for the last two steps, thus requiring only three gene products to complete the
required chemistry (3) (Fig. 1B). Interestingly, the three kineto-plastid protozoan parasites that are known to cause human disease, Trypanosoma brucei, Trypanosoma cruzi, and Leishmania spp., all of which are pyrimidine prototrophs, also employ a bifunctional gene product to catalyze the final two steps in this pathway (20–22). Remarkably, however, the OMPDC domain is at the N terminus of the gene products for all three pathogens, whereas OMPDC is at the C terminus of the mammalian UMPS (Fig. 1B).

Leishmania donovani is the etiologic agent of visceral leishmaniasis, a devastating and invariably fatal disease if untreated. Leishmania spp. are digenetic parasites existing as the flagellated extracellular promastigote in the phlebotomine sandfly vector and as the immotile intracellular amastigote within the phagolysosome of macrophages and other reticuloendothelial cells of the mammalian host. Chemotherapy for visceral leishmaniasis is far from ideal, as the current arsenal of drugs can trigger severe side effects and is often ineffective, possibly due to drug resistance. Thus, the need for developing more efficacious antileishmanial drugs and the characterization and exploitation of novel targets in the parasite that do not exist in the mammalian host is highly desirable.

In this work we investigate the functional importance of LdUMPS to the promastigote form of the parasite and characterize this enzyme structurally and biochemically. We provide evidence that LdUMPS is essential for promastigote viability and present the crystal structure of this bifunctional enzyme. The structure reveals an unusual tetramer that is dependent upon the presence of a ligand for oligomerization. In addition, we have characterized the steady-state kinetics of this enzyme and propose a model to explain the assembly of the catalytically relevant tetramer in vivo. Finally, we discuss the implications of the LdUMPS structure for that of HsUMPS.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—Oligonucleotides were purchased from Integrated DNA Technologies, Inc. (San Diego, CA). All restriction enzymes, Phusion High Fidelity DNA Polymerase, and DNA ligase were purchased from New England Biolabs, Inc. (Beverly, MA). PfuTurbo™ High Fidelity DNA Polymerase was from Agilent Technologies Inc. (Santa Clara, CA). The pBB-CmR-cddB, pCR-HYG, and pCR-PHLEO plasmids used in the construction of the UMPS gene targeting vectors have been described (23). Polyclonal antisera against the purified LdUMPS protein were raised in rabbits as described by Carter and colleagues (24). Guinea pig antibodies against the L. donovani inosine monophosphate dehydrogenase (IMPDH) protein were furnished by Dr. Armando Jardim (McGill University, Montreal, Canada), and the production of this antiserum has been reported (25). All other chemicals and reagents were of the highest quality commercially available.

Isolation of LdUMPS—The UMPS gene was isolated from a L. donovani cosmid library using primers derived from the published L. major genome (20). Details of the cloning strategy will be submitted elsewhere. The UMPS open reading frame (ORF) and ~1 kb of adjacent sequences were sequenced at the Oregon Health and Science University DNA sequencing core facility using an ABI 3130XL sequencer. The UMPS ORF was amplified via polymerase chain reaction (PCR) using PfuTurbo High Fidelity DNA Polymerase with forward primer, 5'-CACC-ATGTCTTTCTTTGCATTTCTACAAGGCG, and reverse primer, 5'-GTGGTTTAGCTTGGTCGCTTTCCACTG-ACC, and inserted into the pET-TOPO-200 Escherichia coli expression vector according to the manufacturer’s instructions.

Overexpression and Purification of L. donovani UMPS and the Monofunctional OMPDC and OPRT Domains—The pET20 vector containing the L. donovani UMPS gene was used to transform BL21(DE3) cells. The cells were grown in LB medium at 37 °C until they reached an A600 of 0.4 at which point the temperature was reduced to 15 °C. After 1 h the cells were induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside and allowed to grow for a further 12 h at 15 °C. The cells were harvested by centrifugation and the pellet was stored at −20 °C prior to purification.

FIGURE 1. Pyrimidine biosynthesis. A, de novo biosynthetic pathway for pyrimidine biosynthesis. B, organization of genes involved in pyrimidine biosynthesis in humans and Leishmania spp. The genes encoding the enzymes responsible for the first three enzymatic steps (carbamoylphosphate synthetase, aspartate carbamoyltransferase, and dihydroorotase) are shown in blue, the gene for dihydroorotate dehydrogenase is shown in green, and the genes encoding the enzymes responsible for the final two steps (OMPDC and OPRT) are shown in red.
Structure of the Bifunctional *L. donovani* UMP Synthase

After thawing the frozen pellet, the cells were lysed by sonication and centrifuged to remove the cellular debris. The supernatant was applied to a nickel-nitriol triacetic acid column that had been pre-equilibrated with buffer containing 300 mM NaCl, 10 mM imidazole, 2 mM dithiothreitol (DTT), 25 mM UMP, and 50 mM sodium phosphate, pH 7.6. The protein on the column was then washed with 20 column volumes of buffer containing 300 mM NaCl, 50 mM imidazole, 2 mM DTT, 25 mM UMP, 50 mM sodium phosphate, pH 7.6, and 10% glycerol (v/v). After the washing step, the protein was eluted with 300 mM NaCl, 2 mM DTT, 25 mM UMP, and 300 mM imidazole, pH 7.6. The protein was split into two fractions, one of which was concentrated to ~20 mg/ml as determined by the method of Bradford (26) and used without further purification, and a second that was further purified and buffer exchanged by gel filtration chromatography. This step involved purifying protein on an ACTA Explorer FPLC with a HiLoad 26/60 Superdex prep grade G200 column running 300 mM NaCl, 5 mM UMP, and 30 mM Tris-HCl, pH 7.6. The eluted protein was concentrated on a centrifugal concentrator to ~20 mg/ml. The protein was determined to be greater than 95% pure by SDS-PAGE. Note that any attempt to reduce the salt concentration below ~300 mM resulted in immediate precipitation of the protein.

The monofunctional *L. donovani* OMPDC and OPRT genes were cloned from the full-length *UMPS* gene and inserted into a pET28 (Novagen)-based vector. The OMPDC (*LdOMPDC*) domain was truncated at Glu260 of the *LdOMPDC* gene and inserted into a pET28 (Novagen)-based vector. The OPRT (PDB code 2P1Z, 31% sequence identity over 90% of the sequence) was easily solved by molecular replacement using MOLREP (27) with the *Plasmodium falciparum* OMPDC structure (PDB code 2F84, 32% sequence identity over 90% of the sequence), as the search model. This structure was refined using alternating cycles of manual model building with Coot (28) and restrained refinement using REFMAC5 (29, 30). Water molecules were added only after the refinement converged.

The *LdOMPDC* dimeric structure was then used as a search model for molecular replacement using the *LdUMPS* data set. A solution was immediately found for the two OMPDC dimeric domains of *LdUMPS*. After fixing these domains, molecular replacement was then successfully used with the *C. difficile* OPRT (PDB code 2P1Z, 31% sequence identity over 86% of the sequence) as a model to locate the OPRT domains of the *LdUMPS*. Refinement was carried out as mentioned above. Ligands were added directly to the corresponding difference density in the *LdUMPS* structure. The refinement statistics are provided in Table 2.

**Enzymatic Assays**—The OMPDC and OPRT activities of *LdUMPS*, *LdOPRT*, and *LdOMPDC* were measured by measuring the decrease in OMP or orotate concentrations, respectively (31, 32), or by HPLC analysis. Before measuring activity, the enzymes were transferred to buffer containing 300 mM NaCl, 2 mM DTT, and 50 mM Tris, pH 8.0. For OPRT activity, the reaction mixture contained 5 mM MgCl₂, 250 μM DTT, 50 mM Tris, pH 8.0, and varying concentrations of either PRPP or orotate. Reactions were initiated by the addition of enzyme at a final concentration of 1 μM and the change in absorbance at 295 nm was monitored.

For OMPDC activity, reactions contained 250 μM DTT, 50 mM Tris, pH 8.0, and varying concentrations of OMP. The reaction was initiated by the addition of enzyme at a final concentration of 1 μM and the change in absorbance at 285 nm was monitored. Alternatively, OMPDC reactions were run as detailed above and then quenched with acetic acid and put on

---

**Table 1**

**Data collection statistics**

<table>
<thead>
<tr>
<th></th>
<th><em>LdOMPDC</em></th>
<th><em>LdUMPS</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Resolution (Å)</td>
<td>1.70</td>
<td>2.99</td>
</tr>
<tr>
<td>Wavelength (Å)</td>
<td>0.987</td>
<td>0.987</td>
</tr>
<tr>
<td>Beam line</td>
<td>24-ID-C</td>
<td>24-ID-C</td>
</tr>
<tr>
<td>Space group</td>
<td>C2</td>
<td>P4,2,2</td>
</tr>
<tr>
<td>a (Å)</td>
<td>101.38</td>
<td>64.72</td>
</tr>
<tr>
<td>b (Å)</td>
<td>98.14</td>
<td>64.72</td>
</tr>
<tr>
<td>c (Å)</td>
<td>62.14</td>
<td>477.60</td>
</tr>
<tr>
<td>β (°)</td>
<td>106.54</td>
<td>90</td>
</tr>
<tr>
<td>No. of reflections</td>
<td>262,430</td>
<td>119,046</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>62,080</td>
<td>34,461</td>
</tr>
<tr>
<td>Average I/σr</td>
<td>24.9 (4.4)</td>
<td>11.3 (2.3)</td>
</tr>
<tr>
<td>Redundancy</td>
<td>4.2 (3.6)</td>
<td>3.5 (1.7)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>98.5 (93.5)</td>
<td>87.7 (39.7)</td>
</tr>
<tr>
<td>Rmerge (%)</td>
<td>6.4 (27.7)</td>
<td>13.1 (37.4)</td>
</tr>
</tbody>
</table>

_r_merg_ = ΣΣ_1/σ_0_ - Σ_0_ / Σ_0_, where _0_ is the mean intensity of the _N_ reflections with intensities _I_ and common indices _h_, _k_, _l_.
Structure of the Bifunctional L. donovani UMP Synthase

TABLE 2
Data refinement statistics

<table>
<thead>
<tr>
<th></th>
<th>LadOMPDC</th>
<th>LadUMPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resolution (Å)</td>
<td>1.70</td>
<td>2.99</td>
</tr>
<tr>
<td>No. of protein atoms</td>
<td>3803</td>
<td>6470</td>
</tr>
<tr>
<td>No. of ligand atoms</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of water atoms</td>
<td>265</td>
<td>11</td>
</tr>
<tr>
<td>No. of reflections in working set</td>
<td>58,930</td>
<td>19,027</td>
</tr>
<tr>
<td>No. of reflections in test set</td>
<td>3,147</td>
<td>996</td>
</tr>
<tr>
<td>R factor</td>
<td>17.2</td>
<td>23.9</td>
</tr>
<tr>
<td>Rfree</td>
<td>21.2</td>
<td>30.4</td>
</tr>
<tr>
<td>Root mean square deviation bonds (Å)</td>
<td>0.028</td>
<td>0.009</td>
</tr>
<tr>
<td>Root mean square deviation angles (°)</td>
<td>2.2</td>
<td>1.1</td>
</tr>
<tr>
<td>Mean B factor (Å²)</td>
<td>23.3</td>
<td>56.9</td>
</tr>
</tbody>
</table>

Ramachandran plot
Most favored (%) | 90.1 | 90.6 |
Additionally allowed (%) | 9.2  | 6.6  |
Generously allowed (%) | 0.7  | 2.8  |
Disallowed (%) | 0.0  | 0     |

* Rfactor = Σ|dobs|(|Fcalc| - |Fc||obs|)/|Fc||obs|, where |Fc||obs| and |Fc||calc| are observed and calculated structure factors, respectively.
* For Rfree, the sum is extended over a subset of reflections (5%) excluded from all stages of refinement.

For 30 min. The samples were centrifuged and loaded onto an Agilent 1100 HPLC with a C-18 reverse-phase column running 10 mM sodium acetate, pH 5.0, isocratically. In all cases, initial rates were calculated from reactions that had proceeded no more than 10% toward completion. Steady-state kinetic parameters were calculated from a fit to the Michaelis-Menten equation.

Size Exclusion Chromatography—The quaternary structure and approximate molecular weight of the bifunctional LadUMPS were determined using size exclusion chromatography. The experiment was carried out on an ACTA Explorer FPLC with a HiLoad 26/60 Superdex prep grade G200 column running 300 mM NaCl, 5 mM UMP, and 30 mM Tris-HCl, pH 7.6. Several proteins of varying molecular mass were used to construct a standard curve. These included HpX T (Klebsiella pneumoniae HIU hydrolase, 44-kDa tetramer), SpNic (Streptococcus pneumoniae nicotinamidase, 84-kDa tetramer), Nc2pur (Neurospora crassa bifunctional purine biosynthetic enzyme, 172-kDa dimer), and GDH (bovine glutamate dehydrogenase, 350-kDa hexamer). The LadUMPS protein was then run using the same apparatus and conditions. Samples of the eluted protein peaks were collected and analyzed by SDS-PAGE. For the experiments with added ligand, the running buffer was supplemented with either 5 mM UMP or 500 μM OMP before the run.

Circular Dichroism Measurements—Immediately prior to taking CD measurements, the enzyme was transferred into buffer containing 10 mM NaCl and 10 mM sodium phosphate, pH 7.6, and used at a final concentration of 0.1 mg/mL. Data were collected separately for the protein alone, for 250 μM UMP alone, and for the enzyme with 250 μM UMP added. The spectrum of the UMP at this concentration was not noticeably different from that of buffer alone. The CD spectra were collected on an AVIV Biomedical (Lakewood, NJ) CD spectrometer, model 202-01. The data were collected at 25 °C from 190 to 260 nm with a 1-nm step size and a 1-nm bandwidth. A 1-mm cell was used in all cases. The programs K2D2 and DicroProt (33, 34) were used for prediction of secondary structure content from the collected data.

Parasite Cell Culture—The wild-type L. donovani subline LdBob (derived from MHOM/SD/00/1S-2D) was originally obtained from Dr. Stephen Beverley (35). LdBob promastigotes were routinely cultured at 26 °C in the M199-based culture medium described by Goyard and colleagues (35) supplemented with 10% fetal bovine serum (Sigma). The medium for propagating the LadUMPS/umsps heterozygotes was supplemented with 50 μg/mL of hygromycin (HYG), whereas the growth medium for the Δumsps null mutants was routinely supplemented with 50 μg/mL of hygromycin (HYG), 50 μg/mL of plasmopine (PHLEO), 250 μM uridine, and 250 μM uracil. Parasite growth experiments were initiated at 1.0 × 10⁵ parasites/ml in Greiner 96-well suspension culture plates (Cellstar®, Dallas, TX). After 96 h, parasites were enumerated using the vital dye alamarBlue® (BioSource International Camarillo, CA) technology (36). Reduction of alamarBlue was monitored at 600 and 570 nm on a Multiskan Ascent plate reader (Thermo Labsystems, Vantaa, Finland). The percent reduction of dye was calculated according to a formula provided by the manufacturer. The greatest reduction was expressed as maximal proliferation, and growth in the absence or presence of one of a variety of pyrimidines was plotted in the histogram form.

Molecular Constructs for Replacement of the LadUMPS Alleles—Targeting vectors for replacement of UMPs by homologous recombination were constructed using the multifragment ligation method described by Fulwiler and colleagues (23). Targeting sequences (TS) were amplified from 5′- and 3′-flanking regions of LadUMPS using L. donovani genomic DNA as a template in PCRs with Phusion DNA polymerase and 5′-TS primers (5′-OPRT SfiA forward, GAggccattggcTCATGGAACCGTGACCACCATCGGTGTTACC, and 5′-ORF SfiB reverse, GAggcctaggcGTTGTTGGGACGCGAGAAAGAC) or 3′-TS primers (3′-OPRT SfiC forward, GAggcctaggcTCACACGCGAGGAG, and 3′-ORF SfiD reverse, GAggcctaggcGGTCCTCTTTCACAGCCGAGGAG). These primers encoded SfiI restriction sites (shown in lowercase) that generate unique overhangs (shown in boldface type) following SfiI digestion that facilitated simultaneous assembly of the complete targeting construct in a single ligation step from its constituent parts (i.e. 5′-targeting sequence, 3′-targeting sequence, drug resistance cassette, and plasmid backbone). The minimal plasmid backbone and expression cassettes conferring resistance to HYG and PHLEO were isolated from donor plasmids pBb-Cmr-ccdB, pCR-HYG, and pCR-PHLEO, respectively, by SfiI digestion and gel purification and combined in a ligation reaction with SfiI-digested and gel-purified UMPs 5′- and 3′-TSs. The UMPs targeting constructs, designated pTRG-UMPS-HYG and pTRG-UMPS-PHLEO, were sequenced to confirm the fidelity of the PCR amplification steps.

Generation of the Δumsps Null Mutants—The Δumsps knockouts were generated by double targeted gene replacement starting with the wild-type L. donovani strain LdBob. In preparation for transfection, the targeting cassettes (drug resistance cassettes flanked by UMPs 5′- and 3′-TSs) were isolated away from the plasmid backbone by cleaving the pTRG-UMPS-HYG and pTRG-UMPS-PHLEO plasmids with PacI followed by gel purification. To create the UMPs/umsps heterozygotes, the HYG and PHLEO targeting cassettes were independently transfected into LdBob promastigotes according to the high voltage protocol of Robinson and Beverley (37), and clones were...
Structure of the Bifunctional L. donovani UMP Synthase

selected on plates containing semi-solid growth medium supplemented with 50 μg/ml of hygromycin or 50 μg/ml of phleomycin. The genotypes of the LUMPS/UMPS heterozygotes were confirmed by PCR using a forward primer (5′-GCGAAGGCAAGAGTAGGTTGAG) corresponding to genomic sequence upstream of the LUMPS 5′-TS and a reverse primer (5′-GTCCTCTATGGTCAACCTTCTTC) within the 5′-flank of the drug resistance cassettes. Independently derived heterozygous clones were then subjected to a second round of transfection to generate the Δumps knock-outs using the reciprocal linearized targeting cassette and the same electroporation protocols employed for producing the heterozygotes. Colonies were picked from semi-solid agarose supplemented with 50 μg/ml of hygromycin, 50 μg/ml of phleomycin, 250 μM uridine, and 250 μM uracil and screened for pyrimidine auxotrophy in pyrimidine-free growth medium. Homozygous umps deficiency was confirmed by PCR and Southern blot analysis as described below.

Southern and Western Blot Analyses—Genomic DNA from wild-type, LUMPS/ums, and Δumps L. donovani was prepared using the DNEasy Blood & Tissue kit (Qiagen) according to the manufacturer’s protocol. Southern blot analysis was performed by standard protocols, and the blots were probed with the LUMPS ORF and a 730-bp fragment corresponding to the LUMPS 5′-TS. Parasites were prepared for Western blotting as follows. 5 × 10⁶ wild-type, LUMPS/ums, and Δumps parasites were subjected to SDS-polyacrylamide gel electrophoresis and transferred to a Immobilon-FL polyvinylidene difluoride membrane (Millipore, Bedford, MA) via a Bio-Rad Trans-Blot SD semi-dry transfer per the manufacturer’s instructions. Membranes were simultaneously probed with rabbit antiseras against purified LdUMPS protein (24) and anti-α-tubulin mouse monoclonal antibody (DM1A) (Calbiochem, San Diego, CA) in Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE) containing 0.3% Tween 20. Binding of primary antibodies was detected with goat anti-mouse IRDye 680 and goat anti-rabbit IRDye 800 CW secondary antibodies (LI-COR Biosciences) and imaged via a LI-COR ODYSSEY Infrared Imager at 700 and 800 nm, respectively.

Immunofluorescence Assay—5 × 10⁶ wild-type L. donovani promastigotes in mid-log phase were attached to poly-l-lysine-coated 4-well Lab-TekII chamber slides (Nalge, Nunc International Corp., Naperville, IL) and fixed with 0.01% glutaraldehyde, 4% paraformaldehyde in PBS for 30 min. Cells were permeabilized with 0.1% Triton X-100 and 0.1% Tween 20 in PBS, blocked with 2% goat serum, and stained with rabbit anti-UMPS polyclonal antiseras (1:2000) in PBS/Tween 20. Primary antibodies were visualized using goat anti-rabbit Oregon Green-conjugated secondary antibodies (1:5000) (Invitrogen). Parasites were also co-stained with guinea pig antibodies raised against L. donovani IMPDH (1:500) and goat anti-guinea pig rhodamine red-conjugated secondary antibodies (1:5000) (Invitrogen) to visualize the IMPDH. All incubations were carried out for 1 h at 25 °C. Confocal microscopy images were acquired on a Zeiss Axiocore 200M inverted microscope (Carl Zeiss Microimaging, Thornwood, NY). Photos were taken with a Zeiss AxioCam MR camera and compiled using Adobe Photoshop Creative Suite 4.

RESULTS AND DISCUSSION

This investigation provides the first detailed functional, biochemical, and structural characterization of the bifunctional enzyme responsible for the final two steps of the pyrimidine biosynthetic pathway in Leishmania. Most previous studies on nucleotide metabolism in Leishmania have focused on the purine nucleotide pathway because Leishmania spp., like all disease-causing parasites in humans, lack the enzymes needed to synthesize purine nucleotides de novo, thus precipitating a great deal of interest in the purine salvage enzymes as possible antiparasitic therapeutic targets (38–40). Conversely, Leishmania are prototrophic for pyrimidines (20) and do not require pyrimidine supplementation for growth in culture, but the genus also accommodates pyrimidine salvage and interconversion enzymes (21, 41, 42). The pyrimidine biosynthetic genes in Leishmania spp. and other trypanosomatids are syntenic and clustered in a contiguous region of 25 kilobases within the genome (see Refs. 21 and 22). This grouping of genes into an operon-like cluster is unusual in Leishmania spp., considering that the genes for virtually all other metabolic pathways are scattered throughout the genome (22, 43). Another notable feature of the pyrimidine biosynthetic pathway in Leishmania spp. is the organization of the gene encoding the enzyme for the final two enzymatic steps. The OPRT and OMPDC-encoding regions are fused, leading to a bifunctional gene product, a phenomenon also observed in mammals. The order of the two domains in Leishmania spp., T. cruzi, and T. brucei (20), however, is reversed relative to that of the bifunctional mammalian UMPs (Fig. 1).

Structure of Monofunctional LdOMPDC and Comparison to Related Structures—To better understand the molecular features of the bifunctional LdUMPS, we determined the structure of this protein by x-ray crystallography. Initially, all attempts to determine the structure by molecular replacement failed, and we were unable to grow diffraction quality selenomethionine-containing crystals for phasing by anomalous diffraction. To overcome this problem, we subcloned the monofunctional LdOMPDC and LdOPRT from the full-length gene and expressed and purified these proteins. Crystals of LdOMPDC were grown and yielded high resolution data that were used to determine its structure by molecular replacement. The structure of LdOMPDC was then used as a model for molecular replacement to position the OMPDC subunits contained within the LdUMPS structure (see discussion of LdUMPS structure below).

The structure of LdOMPDC was determined by molecular replacement using the P. falciparum OMPDC (PDB code 2F84) as a search model (Fig. 2). A dimer was observed in the crystal structure and verified by size exclusion chromatography (data not shown). LdOMPDC displays a (βα)₇α7 fold, typical of other OMPDC orthologs. The overall structure is most similar to bacterial OMPDCs with root mean square deviations ranging from 1.7 to 2.1 Å (supplemental Table S1). The root mean square deviations were 2.2 and 2.6 Å for yeast and human OMPDC, respectively. Comparison of LdOMPDC with HsOMPDC reveals only minor structural differences, the most obvious of which is in a large loop region spanning helices 7 and 8 (Fig. 2B).
The \( \text{LdOMPDC} \) active site contained two regions of density that were modeled as sulfate ions.

**LdOMPDC Active Site Architecture**—As the protein was purified and crystallized in the presence of UMP, it was not surprising to observe electron density in the OMPDC active site of \( \text{LdUMPS} \) corresponding to UMP (Fig. 3A). The \( \text{LdOMPDC} \) active site is near the dimer interface and is comprised of residues from both chains. The organization of the OMPDC active sites is the same in both the monofunctional \( \text{LdOMPDC} \) and the OMPDC domain of \( \text{LdUMPS} \) and is highly similar to those of previously reported OMPDCs (Fig. 3B). Amino acids present in the \( \text{LdOMPDC} \) active site that are known to be completely conserved include Asp\(^{21}\), Lys\(^{49}\), Asp\(^{81}\), Lys\(^{84}\), Asp\(^{88}\), Ile\(^{89}\), Pro\(^{199}\), Gly\(^{200}\) and Arg\(^{229}\) (supplemental Fig. S2). In addition, Ser\(^{137}\), Leu\(^{133}\), and Val\(^{175}\) are also very highly conserved among OMPDC sequences. Of particular importance are the putative catalytic residues, Asp\(^{81}\) and Lys\(^{84}\), which are in positions in the \( \text{LdOMPDC} \) active site that are consistent with their proposed roles in catalysis (10).

**The Structure of \( \text{LdUMPS} \)**—The structure of \( \text{LdUMPS} \) was determined by molecular replacement using \( \text{LdOMPDC} \) and \( \text{Corynebacterium diphtheriae} \) OPRT (PDB code 2P1Z) as search models (Fig. 4A). The asymmetric unit contains two protomers (equivalent to the red/yellow pair or the green/blue pair of protomers shown in Fig. 4B) and the predicted solvent content is \(~50\%\). The linkages between the OMPDC and OPRT domains were clear in both \( \text{LdUMPS} \) monomers; however, the two monomers were not superimposable because of differences in the positioning of the domains relative to one another. The two OMPDC domains in the \( \text{LdUMPS} \) structure form a tight dimer that is homologous to that seen in the monofunctional \( \text{LdOMPDC} \) structure (Figs. 2 and 4). The two domains of each
observed in homologous structures of the monofunctional functional domains in the tetramer are consistent with those in solution was the tetrameric form shown in Fig. 4. The PISA server also predicted that the most stable structure tetramer has a total buried surface area of 20,480 Å² and is an additional interface was observed resulting in dimeric OPRT functional domains in the tetramer are consistent with those observed in homologous structures of the monofunctional enzymes (Fig. 2 and supplemental Fig. S1).

Analysis of the quaternary structure of LdUMPS using the PISA server (44) also predicted that the most stable structure in solution was the tetrameric form shown in Fig. 4B. This tetramer has a total buried surface area of 20,480 Å² and is held together by several important surface interactions. In addition to the OMPDC dimer (2,118 Å² buried surface area) and the OPRT dimer (1,545 Å² buried surface area) interfaces, there is a significant interaction between the two OMPDC domains from each of the two dimers in the LdUMPS tetramer (515 Å² buried surface area). Further surface contact is made between the OPRT dimer and one of the OMPDC domains as the OPRT dimer packs tightly against one face of OMPDC (Fig. 4B).

Ligand-dependent Oligomerization Is Observed by Size Exclusion Chromatography—Given the unusual organization of the tetramer observed in the LdUMPS crystal structure, size exclusion chromatography was carried out to confirm the quaternary structure. The results (Fig. 5A) indicate that this enzyme is found in at least two different oligomeric states. When carried out in buffer containing either 500 μM OMP or 5 mM UMP, two peaks were present. The larger peak corresponded to an apparent molecular mass of ~210 kDa (Fig. 5A). This value is consistent with a tetrameric form, which would have a calculated molecular mass of 200 kDa. A smaller peak is also observed and has an apparent molecular mass of 90 kDa. This peak most likely represents a dimeric form (expected molecular weight of 100 kDa). In a buffer system without added ligand, nearly all of the protein elutes with a molecular weight that corresponds to the dimeric form of the protein. Note that the high concentration of UMP needed to observe the tetrameric species suggests that this ligand is not likely to cause oligomerization in vivo and instead most likely mimicking the structurally similar OMP molecule.

Circular Dichroism Measurements Indicate That Ligand Binding Causes Conformational Change—CD measurements were undertaken in the presence and absence of UMP to investigate whether oligomerization of LdUMPS was accompanied by changes in secondary structure (Fig. 5B). Addition of UMP resulted in a time-dependent change, which corresponded to an increase in helical content and a decrease in disordered regions. The percentage increase in helical content was estimated to be 8–10% based on calculations using the programs K2D2 and DicroProt (33, 34).

Comparison of OPRT Domains of LdUMPS Provides Additional Evidence for Ligand-induced Conformational Change—To date, all of the known crystal structures of OPRT report a similar dimeric form to the one observed in the tetrameric form of LdUMPS (supplemental Fig. S1). In addition, previous structural and biochemical work (45, 46) suggests that interactions from both chains are necessary for catalysis, implying that OPRT is an obligate dimer. The residues from the adjacent chain that participate are on a flexible loop region that is believed to close about the active site to occlude water and prevent unproductive reactions with the oxocarbenium ion intermediate (45, 47, 48). In addition to this loop movement, recent structures of Saccharomyces cerevisiae OPRT reveal an additional conformational change that occurs upon substrate binding (17). A rigid body rotation of a “hood” region was also observed in the complex of S. cerevisiae OPRT with OMP or orotate and PRPP (Fig. 6A). A mechanistic analysis of the same enzyme revealed that the yeast OPRT employs an alternating sites mode of catalysis that is coupled to the structural changes that occur about the active site (45). In the proposed model for catalysis, the binding of substrates to one subunit facilitates the release of products from the other subunit. In this model, the presence and identity of the bound ligands drive the conformational changes that accompany catalysis.

In the structure of LdUMPS, the two OPRT domains present in the asymmetric unit displayed structural differences. Although the flexible loop region believed to occlude solvent
from the adjoining OPRT active site is disordered in the structure of LdUMPS, a superposition of the two OPRT domains revealed a rigid-body shift in a region containing two helices and a loop (Fig. 6A). The observed difference in conformation is similar to the induced asymmetry observed in the S. cerevisiae OPRT structures (17). In addition to the conformational dissimilarity, a comparison of the two OPRT active sites from the two chains in the LdUMPS structure revealed that there were differences in electron density. The active site from the chain in the “closed” conformation contains electron density consistent with a UMP molecule (Fig. 6B). The other LdOPRT active site, however, clearly has little or no ligand present (Fig. 6C). The observed nonequivalence of the OPRT domains and active sites in the LdUMPS structure is consistent with the proposed alternating sites mode of catalysis. It should be noted that, whereas UMP is neither substrate nor product of the OPRT reaction, it differs from OMP only by the presence of the carboxylate and has been shown to be a weak inhibitor of the OPRT reaction (49). It is likely that, due to the high concentration of UMP used in the crystallization conditions, this molecule is binding in place of OMP in the OPRT active site of LdUMPS.

An examination of the protein-ligand interactions within the active site of the closed OPRT domain reveals three residues that are likely to play a role in controlling the conformation of the moving hood region. Phe<sup>276</sup> and Tyr<sup>287</sup> stack with one another and the pyrimidine ring of UMP in the LdOPRT domain (Fig. 6D). In addition, a lysine residue on the loop (Lys<sup>279</sup>) interacts with the phosphate tail of the ligand. The stretch of amino acids that makes up the moving region and includes these three residues is highly conserved among all known OPRT domains (supplemental Fig. S3). It is likely that both of these interactions are necessary to fully close this region about the active site of LdOPRT. This requirement would ensure that both substrates were present before the conformational change occurred. This is consistent with the observed structural change in S. cerevisiae OPRT induced by the binding of both orotate and PRPP or OMP (Fig. 6A).

**Steady-state Kinetics**—To determine how LdUMPS compares kinetically with the isolated, monofunctional enzymes, the OMPDC and OPRT genes were subcloned from full-length LdUMPS, and the respective enzymes were expressed and purified. The point at which the two domains were split was chosen as the midpoint of the linker region between the LdOMPDC and LdOPRT regions. The steady-state kinetic parameters of the full-length and monofunctional enzymes are given in Table 3. The observed $k_{cat}/K_m$ value $\left(1.2 \times 10^6\right)$ for LdOMPDC is consistent with the highly efficient nature of this decarboxylase activity. The efficiency of the OPRT reaction catalyzed by LdUMPS was an order of magnitude slower than that of the OMPDC reaction giving $k_{cat}/K_m$ values of $9.4 \times 10^4$ and $7.9 \times 10^4$ for orotate and PRPP, respectively.

The isolated LdOPRT enzyme was not active under the reaction conditions tested. Note that only a single LdOPRT construct was generated, and it is possible that there is not enough of (or too much of) the linker region present for proper folding and activity. Considering this, we cannot generalize from this negative result, and can conclude only that the particular form of LdOPRT that we expressed is unable to turn over. Combining the isolated LdOMPDC and LdOPRT domains did not substantially alter the kinetics from what was observed for each enzyme tested individually. Adding up to 1 mM UMP to either the OPRT or OMPDC reactions of LdUMPS also did not affect the rates of reaction.

### TABLE 3

**Steady-state kinetic parameters**

<table>
<thead>
<tr>
<th></th>
<th>LdUMPS</th>
<th>LdOMPDC</th>
<th>LdOPRT</th>
<th>LdOMPDC + LdOPRT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{cat}$</td>
<td>$K_m$</td>
<td>$k_{cat}$</td>
<td>$K_m$</td>
</tr>
<tr>
<td>OPRT rxn (Orotate)</td>
<td>$1.2 \times 10^4$</td>
<td>$127$</td>
<td>$–$</td>
<td>$–$</td>
</tr>
<tr>
<td>OPRT rxn (PRPP)</td>
<td>$1.2 \times 10^4$</td>
<td>$151$</td>
<td>$–$</td>
<td>$–$</td>
</tr>
<tr>
<td>OMPDC rxn</td>
<td>$12.9 \times 10^4$</td>
<td>$9.9 \times 10^4$</td>
<td>$12.2 \times 10^4$</td>
<td>$10.6 \times 10^4$</td>
</tr>
</tbody>
</table>

$^a$ ND, no detectable activity.
Although it cannot be ruled out that some part of the N-terminal region of the isolated \textit{LdOPRT} domain is preventing catalysis or proper folding of the enzyme, this region appears unstructured in the \textit{LdUMPS} crystal structure and is quite far from the active site. It is plausible that the \textit{LdOPRT} enzyme is intrinsically unstable and is stabilized by fusion to the \textit{LdOMPDC} domain. Yablonski \textit{et al.} (32) put forth the same explanation for the fusion of the two domains in mammalian UMP synthase. The increased stability of \textit{LdOPRT} may have been the evolutionary driving force for the gene fusion event that brought these domains together. Because the \textit{OPRT} reaction limits the rate of UMP production by limiting the amount of OMP available to OMPDC, an increase in stability of \textit{LdOPRT} leading to increased enzyme efficiency would yield a higher throughput of UMP from orotate and PRPP.

**Proposed Model for the Assembly of the \textit{LdUMPS} Tetramer**—Given the structural and biochemical data presented, a model for the assembly of the catalytically active form of \textit{LdUMPS} is proposed (Fig. 7). The first step after expression is formation of a tight dimer at the OMPDC domains. This is consistent with the gel filtration data indicating that in the absence of a ligand, \textit{LdUMPS} was observed entirely as a dimer. This dimer is stable in solution but is not likely to catalyze the \textit{OPRT} reaction because the \textit{OPRT} obligate dimer has yet to form. As indicated by CD and gel filtration, the presence of a ligand causes a conformational change to occur. The structure of \textit{LdUMPS} indicates that this change takes place within the \textit{OPRT} domain. It is this ligand-dependent conformational change that likely promotes dimerization at the \textit{OPRT} domain, leading to a complete \textit{LdUMPS} tetramer. The tetramer would be catalytically competent for both OMPDC and \textit{OPRT} reactions.

The physical association and complexation of \textit{OPRT} and OMPDC is not unique to \textit{Leishmania} \textit{spp}. Recent work on \textit{P. falciparum}, which encode and express separately the two enzymes, has shown that OMPDC and \textit{OPRT} associate into a heterotetrameric complex for optimal catalytic efficiency (50). In addition, Traut and Jones (32, 51, 52) used gel filtration and sedimentation experiments to explore the interconvergence of different forms of mammalian UMPS. They found that this enzyme can adopt several different species, including at least two forms with molecular weights equal to or greater than that of a dimer. They determined that adding several compounds, including orotate and PRPP together, OMP or UMP, they could promote the formation of the larger molecular weight species. An earlier report also found that human UMPS could assume monomeric, dimeric, and tetrameric forms in the presence of various effector molecules (53).

**Implications for Human UMP Synthase**—Despite the difference in gene order of the \textit{LdUMPS} and mammalian UMP synthase, it is possible that the human enzyme may adopt a similar domain organization. Superposition of the human OMPDC and \textit{OPRT} structures with that of \textit{LdUMPS} reveals that the \textit{OPRT} C terminus and OMPDC N terminus are accessible to one another and could be joined by a linker region of an appropriate length (supplemental Fig. S4). Considering that approximately 20 residues of the linker region are missing from the partial structures of UMPS (PDB codes 2EAW, 2QCC, 3G3D, 3EWU, 2WNS and related structures) and that secondary structure prediction algorithms predict this region to be disordered and solvent-exposed (PsiPRED (54), JPred (55), and PredictProtein (56)), it is entirely feasible that mammalian UMPS could adopt a structure similar to that of \textit{LdUMPS}. However, further structural studies are required to determine whether this structural association of \textit{OPRT} and OMPDC is a conserved motif and to what extent this conservation occurs.

**Molecular Characterization of the \textit{umps} Knock-out**—To assess whether \textit{LdUMPS} is nutritionally indispensable for \textit{L. donovani} promastigotes grown in culture, UMPS gene function was tested via the construction of a \textit{umps} knock-out by double targeted gene replacement. Each of the two wild-type UMPS gene copies was sequentially replaced with a linearized drug resistance cassette encompassing a dominant selectable marker. Southern blotting of the double knock-out clone and its heterozygous parent revealed the expected sequential losses of the wild-type alleles in the UMPS/\textit{umps} and \textit{umps} lines.
The effects of the medium in the absence of a pyrimidine supplement. The mutant could not proliferate in wild-type growth because the mutant could not proliferate in wild-type growth

FIGURE 9. Growth of Δumps parasites in various pyrimidine sources. The ability of wild-type (black bars) and Δumps (hashed bars) parasites to grow in medium containing orotic acid or a pyrimidine supplement was ascertained as described under “Experimental Procedures.” All supplements were present at a concentration of 100 μM, except where indicated. Data are plotted in histogram form as a percentage of maximal growth against mouse α-tubulin (24). Lanes 1–3 in all panels refer to DNA or protein obtained from wild-type, LdUMPS/umps, and Δumps cells, respectively.

(1) Western blot analysis confirmed the lack of UMPs protein in the Δumps line.

Growth Phenotype of the Δumps Knock-out—Because UMPs is a bifunctional protein that converts orotic acid to UMP, the effects of the Δumps lesion on parasite viability were examined. The Δumps knock-out was auxotrophic for pyrimidines because the mutant could not proliferate in wild-type growth medium in the absence of a pyrimidine supplement. The pyrimidine auxotrophy could be overcome by the addition of uracil, uridine, deoxyuridine, cytidine, or deoxycytidine (Fig. 9). Growth of the null mutant was more robust in medium containing either uracil or its nucleosides than in medium supplemented with cytidine or deoxycytidine, presumably because cytidine/deoxycytidine deamination is rate-limiting for growth in medium to which the cytosine-containing nucleosides were added. As expected, the knock-out parasites were unable to overcome their pyrimidine auxotrophy with orotic acid, cytosine, thymine, or thymidine. The relative growth of the null mutant in orotic acid or various pyrimidine nucleobases and nucleosides could not be ascribed to toxicity, because the growth of wild-type L. donovani, which are prototrophic for pyrimidines, was unaffected by the addition of any of these compounds to the culture medium. The growth phenotype of the Δumps knock-out demonstrates that LdUMPS is an essential protein in L. donovani promastigotes in culture and that the sole functional role of this bifunctional protein is to synthesize pyrimidine nucleotides. Whether or not the LdUMPS is essential for the promastigote in the sandfly vector or for the infectious form of the parasite in mammals, i.e. the amastigote, remains to be evaluated.

Subcellular Localization of LdUMPS—Although human OPRT and OMPDC are cytosolic enzymes, these proteins were found to copurify with known glycosomal enzymes in T. brucei, T. cruzi, and Leishmania mexicana (57). The COOH-terminal tripeptide of the LdUMPS is Ser-Lys-Leu, the archetypical topogenic signal for directing leishmanial proteins to the glycosome (supplemental Fig. S3 (58, 59)), a peroxisome-like microbody unique to Leishmania and related parasites, that accommodates a variety of nutritional enzymes (60). To establish whether the LdUMPS is confined to the glycosomal compartment, the intracellular milieu in which the enzyme is located was determined by indirect immunofluorescence using the previously characterized polyclonal antisera generated to purified LdUMPS (21). This analysis revealed a punctate staining pattern consistent with a glycosomal milieu (Fig. 10). An analogous experiment with antibodies against the L. donovani IMPDH, a known glycosomal marker (25), and a merge of the two fluorescent images clearly indicated that LdUMPS co-localized with IMPDH.

In this study we present the first structural results for the physical association of the OPRT and OMPDC enzymes in any organism and provide evidence for a ligand-dependent confor-
mational change and oligomerization of LdUMPS. In addition, we have demonstrated that the bifunctional LdUMPS is essential for promastigote viability in intact L. donovani and is localized to the parasitemic glycosome. The structural and biochemical data presented provides new insights into the cooperative function of these two important enzymes.

Acknowledgments—We thank the staff at the Northeastern Collaborative Access Team (NE-CAT) beamlines for advice with data collection and processing. Data collected at the NE-CAT beamlines of the Advanced Photon Source are supported by award RR-15301 from the National Center for Research Resources at the National Institutes of Health. Use of the Advanced Photon Source is supported by the United States Department of Energy, Office of Basic Energy Sciences, contract number DE-AC02-06CH11357. We acknowledge Cynthia Kinsland of the Protein Production Facility in the Department of Chemistry and Chemical Biology for help with molecular biology and Leslie Kinsland for help with manuscript preparation.

REFERENCES

The *Leishmania donovani* UMP Synthase Is Essential for Promastigote Viability and Has an Unusual Tetrameric Structure That Exhibits Substrate-controlled Oligomerization

Jarrod B. French, Phillip A. Yates, D. Radika Soysa, Jan M. Boitz, Nicola S. Carter, Bailey Chang, Buddy Ullman and Steven E. Ealick

doi: 10.1074/jbc.M111.228213 originally published online April 19, 2011

Access the most updated version of this article at doi: 10.1074/jbc.M111.228213

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2011/04/19/M111.228213.DC1

This article cites 59 references, 13 of which can be accessed free at
http://www.jbc.org/content/286/23/20930.full.html#ref-list-1