The \textit{Leishmania donovani} UMP Synthase is Essential for Promastigote Viability and has an Unusual Tetrameric Structure that Exhibits Substrate-Controlled Oligomerization *

Jarrod B. French$^1$, D. Radika Soysa$^2$, Phillip A. Yates$^2$, Jan M. Boitz$^2$, Nicola S. Carter$^2$, Bailey Chang$^1$, Buddy Ullman$^2$, Steven E. Ealick$^1$

$^1$Department of Chemistry and Chemical Biology, Cornell University, Ithaca, New York 14853

$^2$Department of Biochemistry and Molecular Biology, Oregon Health & Science University, Portland, Oregon 97239

Address correspondence to: Steven E. Ealick, 120 Baker Lab, Cornell University, Ithaca, NY 14853-1301. Fax: (607) 255-1227; E-mail: see3@cornell.edu

The final two steps of \textit{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, while 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. \textit{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 \textit{L. donovani} UMPS (\textit{Ld}UMPS) is an essential enzyme in promastigotes and that it is sequestered in the parasite glyosome. We also present the crystal structure of the \textit{Ld}UMPS 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 \textit{Ld}UMPS is controlled by product binding at the OPRT active site. We propose a model for the assembly of the catalytically relevant \textit{Ld}UMPS 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 \textit{de novo} from small molecule metabolites and by salvage of preformed pyrimidine bases. Some microorganisms, such as \textit{Giardia lamblia} and \textit{Entamoeba histolytica} (4), lack the \textit{de novo} pathway and rely exclusively on pyrimidine salvage for their pyrimidine needs. For higher organisms, the relative contribution of the \textit{de novo} and salvage pathways is dependent upon cell type and stage of development. The \textit{de novo} pathway is important in proliferating cells and, as such, is upregulated 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 \textit{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) 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 ($k_{\text{cat}}/k_{\text{non}}$) (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 (\textit{Hs}UMPS), 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-fluorouracil (16). There are also several available structures of...
OPRT (17-19) including a recent structure of the human OPRT domain of UMP synthase (PDB ID 2WNS).

While 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 kinetoplastid 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, while 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-cdB, pCR-HYG, and pCR-PHLEO plasmids used in the construction of the UMPs gene targeting vectors have been described (23). Polyclonal antisera against purified LsUMPS 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 an 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 & Science University DNA sequencing core facility using an ABI 3130XL sequencer. The UMPs ORF was amplified via the polymerase chain reaction (PCR) using PfuTurbo™ High-Fidelity DNA Polymerase with forward primer 5'-CACCATGTCTTTCTTGATCTCCTCAACGA GCG and reverse primer 5'-GTGGTTATAGCTTGCTCTGCTTCCACTGAC C and inserted into the pET-TOPO-200 E. coli expression vector according to the manufacturer’s instructions.

Overexpression and Purification of L. donovani UMPs and the Monofunctional OMPDC and OPRT Domains – The pET200 vector containing the L. donovani UMPs gene was used to transform BL21(DE3) cells. The cells were

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grown in LB media at 37 °C until they reached an O.D.₆₀₀ of 0.4 at which point the temperature was reduced to 15 °C. After one 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.

After thawing the frozen pellet, the cells were lysed by sonication and centrifuged to remove the cellular debris. The supernatant was applied to an Ni-NTA 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 approximately 20 mg/mL as determined by the method of Bradford (26) and used without further purification, and a second which 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 approximately 20 mg/mL as determined by the method of Bradford (26) and 90% pure by SDS-PAGE. The protein was transferred into 30 mM NaCl and 10 mM Tris, pH 7.6 using an Econo-Pac 10DG desalting column (BioRad) and were determined to be greater than 95% pure by SDS-PAGE.

Crystallization of LdUMPS and Monofunctional LdOMPDC – Crystallization was performed using the hanging drop vapor diffusion method at 18 °C. For LdUMPS, protein was used at 10 mg/mL in buffer containing 300 mM NaCl, 25 mM UMP, 2 mM DTT and 300 mM imidazole pH 7.6. Optimization of crystallization conditions led to crystals that grew in 30 - 34 % PEG-400, 200 - 300 mM NaCl and 0.1 M HEPES 6.5. The crystals were approximately cubic in shape and were cryoprotected using the mother liquor without further supplementation. Slightly larger and higher quality crystals were grown by microseeding into the above-mentioned solution.

LdOMPDC at a concentration of 20 mg/mL was used in buffer containing 30 mM NaCl and 10 mM Tris, pH 7.6. Large crystals grew after approximately 1 month in 1.6 M ammonium sulfate, 10% v/v 1,4-dioxane and 0.1 M MES, pH 6.5. The crystals were cryoprotected using a short soak in 2.0 M ammonium sulfate, 10% v/v 1,4-dioxane and 0.1 M MES, pH 6.4.

Data Collection, Structure Determination and Refinement – The data for both LdUMPS and the monofunctional LdOMPDC were collected at 100 °C at the NE-CAT beam line 24-ID-C at the Advanced Photon Source. Data were collected over 200° using a 0.5° oscillation range using an ADSC Quantum 315 detector. The presence of a long unit cell axis for LdUMPS limited data collection to approximately 2.9 Å in order to clearly resolve reflections. The data collection statistics are provided in Table 1.

Initial attempts to solve the LdUMPS structure using molecular replacement failed for all available OPRT or OMPDC structures as models. Additionally, we were unable to grow crystals of diffraction quality using selenomethionine-containing LdUMPS. The monofunctional OMPDC protein, however, was easily solved by molecular replacement using MOLREP (27) with the P. falciparum OMPDC structure (PDB ID 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 *Ld*UMPS. After fixing these domains, molecular replacement was then successfully used with the *C. diptheriae* OPRT (2P1Z, 31% sequence identity over 86% of the sequence) as a model to locate the OPRT domains of the *Ld*UMPS. Refinement was carried out as mentioned above. Ligands were added directly to the corresponding difference density in the *Ld*UMPS structure. The refinement statistics are provided in Table 2.

**Enzymatic Assays** — The OMPDC and OPRT activities of *Ld*UMPS, *Ld*OPRT and *Ld*OMPDC 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 MgCl$_2$, 250 µM DTT, and 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 ice for thirty 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% towards 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 *Ld*UMPS 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 weight were used in order to construct a standard curve. These included HpxT (*K. pneumoniae* HIU hydrolase, 44 KDa tetramer), SpNic (*S. pneumoniae* nicotinamidase, 84 KDa tetramer), Nc2Pur (*Neurospora crassa* bifunctional purine biosynthetic enzyme, 172 KDa dimer), and GDH (bovine glutamate dehydrogenase, 350 KDa hexamer). The *Ld*UMPS 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 *Ld*Bob (derived from MHOM/SD/00/1S-2D) was originally obtained from Dr. Stephen Beverley (35). *Ld*Bob 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 Aldrich, St. Louis, MO). The medium for propagating the UMPS/umps heterozygotes was supplemented with 50 µg/mL hygromycin (HYG), while the growth medium for the *umps null* mutants was routinely supplemented with 50 µg/mL HYG 50 µg/mL phleomycin (PHLEO), 250 µM uridine, and 250 µM uracil. Parasite growth experiments were initiated at 1.0 X 10$^5$ parasites per mL in Greiner 96 well suspension culture plates (Cellstar®, Dallas/Fort Worth, 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 histogram form.

Molecular Constructs for Replacement of the \textit{LdUMPS} Alleles – Targeting vectors for replacement of \textit{UMPS} by homologous recombination were constructed using the multi-fragment ligation method described by Fulwiler and colleagues (23). Targeting sequences (TS) were amplified from 5’- and 3’-flanking regions of \textit{LdUMPS} using \textit{L. donovani} genomic DNA as a template in PCRs with Phusion DNA polymerase and 5’-TS primers (5’-OPRT SfiA F - GAggccacagtggcTATGACATGCGCTGTA CC; and 5’-OPRT SfiB R - GAggccacagtggcGTGTGGAGCGAGAGG AAGAC) or 3’-TS primers (3’-OPRT SfiC F - GAggcctggctgacGACACACGCGAG AG; and 3’-OPRT SfiD R - GAggcctggctgacTCTCTCTTACAGCCAGTGT TGC). These primers encoded SfiI restriction sites (shown in lower case) 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 cassette 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 \textit{UMPS} 5’- and 3’-TSs. The \textit{UMPS} targeting constructs, designated pTRG-\textit{UMPS-HYG} and pTRG-\textit{UMPS-PHLEO}, were sequenced to confirm the fidelity of the PCR amplification steps.

\textit{Generation of the \textit{Aumps} Null Mutants} – The \textit{Aumps} knockouts were generated by double targeted gene replacement starting with the wild type \textit{L. donovani} strain \textit{LdBob}. In preparation for transfection, the targeting cassettes (drug resistance cassettes flanked by \textit{UMPS} 5’- and 3’-TSs) were isolated away from the plasmid backbone by cleaving the pTRG-\textit{UMPS-HYG} and pTRG-\textit{UMPS-PHLEO} plasmids with Pac I followed by gel purification. To create the \textit{UMPS/umps} heterozygotes, the HYG and PHLEO targeting cassettes were independently transfected into \textit{LdBob} promastigotes according to the high voltage protocol of Robinson and Beverley (37), and clones were selected on plates containing semi-solid growth medium supplemented with 50 \(\mu\)g/mL hygromycin or 50 \(\mu\)g/mL phleomycin. The genotypes of the \textit{UMPS/umps} heterozygotes were confirmed by PCR using a forward primer (5’-GCGAAGGCAAGGATGGTGAG) corresponding to genomic sequence upstream of the \textit{UMPS} 5’-TS and a reverse primer (5’-CGTCCTCATGGAACACCATCTCTTC) 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 \textit{Aumps} knockouts 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 \(\mu\)g/mL hygromycin, 50 \(\mu\)g/mL phleomycin, 250 \(\mu\)M uridine, and 250 \(\mu\)M uracil and screened for pyrimidine auxotrophy in pyrimidine-free growth medium. Homozygous \textit{umps} deficiency was confirmed by PCR and Southern blot analysis as described below.

\textit{Southern and Western Blot Analyses} – Genomic DNA from wild type, \textit{UMPS/umps}, and \textit{Aumps L. donovani} was prepared using the DNAeasy Blood & Tissue kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol. Southern blot analysis was performed by standard protocols, and the blots were probed with the \textit{UMPS} ORF and a 730 bp fragment corresponding to the \textit{UMPS} 5’-TS. Parasites were prepared for western blotting as follows. \(5 \times 10^6\) wild type, \textit{UMPS/umps}, and \textit{Aumps} 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 brochure. Membranes were simultaneously probed with rabbit antisera against purified \textit{LdUMPS} protein (24) and anti-\(\alpha\)-tubulin mouse monoclonal antibody (DM1A) (Calbiochem, San Diego, CA) in Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln,
NE) containing 0.2% 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 nm and 800 nm, respectively.

**Immunofluorescence Assay** - 5 X 10^6 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 antisera (1:2000) in PBS-Tween-20. Primary antibodies were visualized using goat anti-rabbit Oregon Green-conjugated secondary antibodies (1:5000) (Invitrogen, Carlsbad, CA). Parasites were also co-stained with guinea pig antibodies raised against *L. donovani* IMP dehydrogenase (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 Axiovert 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 metabolism 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 (http://tritrypdb.org/tritrypdb/, (21,22)). 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 genes in *Leishmania* spp., *Trypanosoma cruzi*, and *T. brucei* (20), however, is reversed relative to that of the bifunctional mammalian UMPS and is unique in nature (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 in order 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 *Plasmodium falciparum* OMPDC (PDB ID 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)8 fold, typical of other OMPDC orthologs. The overall structure is most similar to
bacterial OMPDCs with RMSDs ranging from 1.7 to 2.1 Å (Table S1). The RMSDs 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 *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 *LdUMPS* corresponding to UMP (Fig. 3A). The *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 *LdOMPDC* and the OMPDC domain of *LdUMPS* and is highly similar to those of previously reported OMPDC’s (Fig. 3B). Amino acids present in the *LdOMPDC* active site that are known to be completely conserved include Asp21, Lys49, Asp81, Lys84, Asp88, Ile89, Pro199, Gly200 and Arg229 (Fig. S2). In addition, Ser137, Leu133 and Val175 are also very highly conserved among OMPDC sequences. Of particular importance are the putative catalytic residues, Asp81 and Lys84, which are in positions in the *LdOMPDC* active site that are consistent with their proposed roles in catalysis (10).

**The Structure of LdUMPS** – The structure of *LdUMPS* was determined by molecular replacement using *LdOMPDC* and *C. diphtheriae* OPRT (PDB ID 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 approximately 50%. The linkages between the OMPDC and OPRT domains were clear in both *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 *LdUMPS* structure form a tight dimer that is homologous to that seen in the monofunctional *LdOMPDC* structure (Figs. 2 and 4). The two domains of each chain, while covalently linked in the structure, do not make any significant domain-domain contacts (Fig. 4A). When twofold crystallographic symmetry is considered, however, an additional interface was observed resulting in dimeric OPRT functional domains (Fig. 4B). The dimeric *LdOMPDC* and *LdOPRT* functional domains in the tetramer are consistent with those observed in homologous structures of the monofunctional enzymes (Figs. 2 and 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 weight of approximately 210 kDa (Fig. 5A). This value is consistent with a tetrameric form, which would have a calculated molecular weight of 200 kDa. A smaller peak is also observed and has an apparent molecular weight 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 is instead most likely mimicking the structurally similar OMP molecule.

**Circular Dichroism Measurements Indicate that Ligand Binding Causes Conformational
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 (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 in order to occlude water and prevent unproductive reactions with the oxocarbenium ion intermediate (45,47,48). In addition, a lysine residue on the loop (Lys279) interacts with the phosphate tail of the ligand. The observed nonequivalence of the OPRT domains active sites in the LdUMPS structure is consistent with the proposed alternating sites mode of catalysis. It should be noted that, while 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. Phe276 and Tyr287 stack with one another and the pyrimidine ring of UMP in the LdOPRT domain (Fig. 6D). In addition, a lysine residue on the loop (Lys279) interacts with the phosphate tail of the ligand. The stretch of amino acids that makes up the moving 'hood' region is disordered in the structure of LdUMPS. The observed nonequivalence of the OPRT domains active sites in the LdUMPS structure is consistent with the proposed alternating sites mode of catalysis. It should be noted that, while 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.

Steady-State Kinetics

In order 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 mid-point 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 ($1.2 \times 10^8$) 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 $7.9 \times 10^6$ 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 effect the rates of reaction.

While it cannot be ruled out that some part of the N-terminal region of the isolated LdOPRT domain is preventing catalysis or proper folding of the enzyme, this region appears unstructured in the LdUMPS crystal structure and is quite far from the active site. It is plausible that the LdOPRT enzyme is intrinsically unstable and is stabilized by fusion to the LdOMPDC domain. Yablonski et al. put forth the same explanation for the fusion of the two domains in mammalian UMP synthase (32). The increased stability of LdOPRT may have been the evolutionary driving force for the gene fusion event that brought these domains together. Since the OPRT reaction limits the rate of UMP production by limiting the amount of OMP available to OMPDC, an increase in stability of LdOPRT leading to increased enzyme efficiency would yield a higher throughput of UMP from orotate and PRPP.

Proposed Model for the Assembly of the LdUMPS Tetramer – Given the structural and biochemical data presented, a model for the assembly of the catalytically active form of 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, LdUMPS was observed entirely as a dimer. This dimer is stable in solution but is not likely to catalyze the OPRT reaction because the 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 LdUMPS indicates that this change takes place within the OPRT domain. It is this ligand-dependent conformational change that likely promotes dimerization at the OPRT domain, leading to a complete LdUMPS tetramer. The tetramer would be catalytically competent for both OMPDC and OPRT reactions.

The physical association and complexation of OPRT and OMPDC is not unique to Leishmania spp. Recent work on Plasmodium falciparum, which encode and express separately the two enzymes, has shown that OMPDC and 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 interconversion 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 LdUMPS and mammalian UMP synthase, it is possible that the human enzyme may adopt a similar domain organization. Superposition of the human OMPDC and OPRT structures with that of LdUMPS reveals that the OPRT C-terminus and OMPDC N-terminus are accessible to one another and could be joined by a linker region of an appropriate length (Fig. S5). Considering that approximately twenty residues of the linker region are missing from the partial structures of UMPS (PDB IDs 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),
the growth of wild type nucleosides could not be ascribed to toxicity, since acid or various pyrimidine nucleobases and The relative growth of the null mutant in orotic with orotic acid, cytosine, thymine, or thymidine. unable to overcome their pyrimidine auxotrophy added. As expected, the knockout parasites were unable to overcome their pyrimidine auxotrophy to which the cytosine-containing nucleosides were deamination is rate-limiting for growth in medium to which the cytosine-containing nucleosides were added. As expected, the knockout 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, since 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 knockout 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.

Molecular Characterization of the Δumps Knockout – In order to assess whether LdUMPS is nutritionally indispensable for L. donovani promastigotes grown in culture, UMPs gene function was tested via the construction of a Δumps knockout 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 knockout clone and its heterozygous parent revealed the expected sequential losses of the wild type alleles in the UMPs/umps and Δumps lines (Fig. 8). Western blot analysis confirmed the lack of UMPs protein in the Δumps line.

Growth Phenotype of the Δumps Knockout – 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 knockout was auxotrophic for pyrimidines since 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 knockout 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, since 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 knockout 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 – While human OPRT and OMPDC are cytosolic enzymes, these proteins were found to copurify with known glycosomal enzymes in T. brucei, T. cruzi, and L. mexicana (57). The COOH-terminal tripeptide of the LdUMPS is Ser-Lys-Leu, the archetypical topogenic signal for directing leishmanial proteins to the glycosome (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 in which the enzyme is located was determined by indirect immunofluorescence using the previously characterized polyclonal antisera generated to purified LdUMPS (21). An analogous experiment with antibodies against the L. donovani inosine monophosphate dehydrogenase (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 conformational 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 parasite glycosome. The structural and biochemical data presented provides new insights into the cooperative function of these two important enzymes.
REFERENCES

FOOTNOTES

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The atomic coordinates and structure factors (codes 3QW3 and 3QW4) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

The abbreviations used are: OMPDC, orotidine 5-monophosphate decarboxylase; OPRT, orotate phosphoribosyltransferase; PRPP, 5-phosphoribosyl-1-pyrophosphate; LdUMPS, Leishmania donovani OMPDC-OPRT; UMP, uridine 5' monophosphate; UMPS, uridine 5'-monophosphate synthase; CPS, carbamoylphosphate synthetase; ACT, aspartate carbamoyltransferase; DHO, dihydroorotase; DHOD, dihydroorotate dehydrogenase; CAD, CPS-ACT-DHO; IMPDH, L. donovani inosinate dehydrogenase; HYG, hygromycin; PHLEO, phleomycin.
FIGURE LEGENDS

Fig. 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 (CPS, ACT, and DHO) are shown in blue, the gene for DHOD is shown in green and the genes encoding the enzymes responsible for the final two steps (OMPDC and OPRT) are shown in red.

Fig. 2. Structure of LdOMPDC. A) Ribbon diagram of the structure of monofunctional LdOMPDC with sulfate molecules (space-filling) in the active site. N and C mark the N- and C-termini of the protein. Helices are shown colored red, while β-strands are shown colored yellow. B) Comparison of LdOMPDC dimer (green and red/yellow) to the human OMPDC dimer (blue and red/yellow).

Fig. 3. The OMPDC domain of LdUMPS. A) Ribbon diagram of the OMPDC domain of LdUMPS showing UMP bound in the active sites. The electron density shown is from an F₀ – Fₐ map calculated before adding the ligand and contoured at 2.5σ. B) Stereodiagram of the active site of the OMPDC domain of LdUMPS. The carbon atoms of residues from different OMPDC chains are colored in yellow and cyan respectively, and the UMP molecule is shown with green carbon atoms. In both cases, oxygen atoms are colored red and nitrogen atoms are colored blue.

Fig. 4. Structure of LdUMPS. A) Ribbon diagram of the LdUMPS protomer with the two domains labeled. Helices are shown colored red, while β-strands are shown colored yellow. B) LdUMPS tetramer with the four chains shown in red, yellow, green and blue and the OPRT and OMPDC dimers highlighted by green and blue surface representation, respectively. The tetramer was constructed with two copies of the asymmetric unit related by a twofold rotation. The yellow/red pair and the blue/green pair each represent a single copy of the asymmetric unit.

Fig. 5. Ligand-dependent oligomerization of LdUMPS. A) Size exclusion chromatography of LdUMPS run in buffer without added ligand (red solid line), with 5 mM UMP added (blue dashed line), and with 500 µM OMP added (black dotted line). A UMP peak appears in all chromatograms as a result of UMP that is present in the purification of LdUMPS. B) CD spectra of LdUMPS without added UMP (black dashed line) and at two time points after adding UMP (red and blue solid lines).

Fig. 6. The OPRT domain of LdUMPS. A) Superposition of apo and ligand-bound S. cerevisiae OPRT (17) and of the two OPRT domains from the structure of LdUMPS. B) and C) Active site of OPRT from the two different chains found in the asymmetric unit of the structure of LdUMPS. UMP is modeled into the density for B), and shown in a similar location in C). The electron density shown is from an F₀ – Fₐ map calculated before adding the ligand and contoured at 2.5σ. D) A superposition of the two different OPRT chains shows the residues believed to be responsible for closing the 'hood' region about the active site. The 'closed' conformation of the enzyme is shown in green, while the 'open' conformation is shown in blue and UMP is shown colored grey. Lys279 is not shown for the chain in the 'open' conformation as it is not visible in the LdUMPS structure.

Fig. 7. Model for assembly of the biologically active LdUMPS tetramer. After expression, the bifunctional LdUMPS forms a tight dimer at the OMPDC domain. Ligand binding causes a conformational change in the OPRT domain (shown in red) which facilitates the dimerization at the OPRT domain, leading to the observed tetrameric form.

Fig. 8. Southern and western blot analysis of Δumps parasites. A) Genomic DNA prepared from wild type, LdUMPS/umps, and Δumps parasites was digested with Pvu II and hybridized to a 1.37 kb probe from the LdUMPS ORF and B) to a 0.73 kb probe from the 5'-flanking region of LdUMPS. C) A
western blot of wild type, LdUMPS/umps, and Δumps cell lysates was fractionated on an SDS-polyacrylamide gel and probed with monospecific antisera raised against the purified LdUMPS protein and monoclonal antibody directed against mouse α-tubulin (24). Lanes 1, 2 and 3 in all panels refer to DNA or protein obtained from wild type, LdUMPS/umps, and Δumps cells, respectively.

Fig. 9. Growth of Δumps promastigotes 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 in Materials and Methods. 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 versus the supplement added to the normal culture medium. No pyr designates no pyrimidine added, and Oro2 mM indicates that orotic acid was added to the parasites at a final concentration of 2 mM. Each result provided is the mean and standard deviation of quadruplicate determinations.

Fig. 10. Subcellular localization of LdUMPS. Exponentially growing L. donovani promastigotes were fixed and processed as described in the experimental procedures section prior to incubation with rabbit anti-LdUMPS and guinea pig anti-IMPDH antibodies. Primary antibodies were visualized at 488 nm with (A) Oregon Green-conjugated goat anti-rabbit IgG or (B) at 594 nm with Rhodamine Red-conjugated goat anti-guinea pig IgG secondary antibodies. (C) The images in panels A and B were merged to demonstrate colocalization of LdUMPS and IMPDH. Confocal images were acquired on a Zeiss Axiovert 200M inverted microscope. The green signal is LdUMPS, while the red signal shows the localization of LdIMPDH.
Table 1. Data Collection Statistics.

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Numbers in parentheses correspond to the highest resolution shell

\[ R_{sym} = \frac{\sum |I_i - \langle I \rangle|}{\sum \langle I \rangle}, \]  where \( \langle I \rangle \) is the mean intensity of the \( N \) reflections with intensities \( I_i \) and common indices \( h, k, l \).
Table 2. Data Refinement Statistics.

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Ramachandran plot

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<sup>a</sup> R factor = \( \sum_{hk} \frac{\| F_{obs} \| - k \| F_{calc} \|}{\| \sum_{hk} F_{obs} \|} \), where \( F_{obs} \) and \( F_{calc} \) are observed and calculated structure factors respectively.

<sup>b</sup> For R<sub>free</sub>, the sum is extended over a subset of reflections (5%) excluded from all stages of refinement.
Table 3. Steady-state Kinetic Parameters

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ND: no detectable activity
Figure 1
Figure 2.
Figure 3.
Figure 4.
Figure 5.
Figure 6.
Figure 7.
Figure 8.
Figure 9.
Figure 10.
The *Leishmania donovani* UMP synthase is essential for promastigote viability and has an unusual tetrameric structure that exhibits substrate-controlled oligomerization.

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

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