Characterization of a novel *Leishmania* guanosine 5'-monophosphate reductase

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Abstract

Leishmania parasites are reliant on salvage mechanisms to acquire purines from the extracellular environment. GMP reductase (GMPR) catalyzes the conversion of GMP to IMP, an integral reaction for maintaining purine nucleotide balance. Enzymatically active *L. major* GMPR (LmGMPR) has been cloned, expressed and purified. The LmGMPR gene complements GMPR deficiency in *E. coli* strains. Quaternary structure analysis indicates that LmGMPR forms tetramers and higher order complexes under reducing conditions. Kinetic assays reveal that the enzyme deviates from hyperbolic behaviour with regard to GMP but conforms to typical Michaelis-Menten kinetics for NADPH. Sequence analysis indicates that LmGMPR contains CBS domains and an MPA binding site. MPA competes for the NADPH binding site with a K_i of 20 μM. ATP and GTP regulate enzymatic activity through inhibition and activation, respectively. This data indicates that LmGMPR is a novel enzyme that performs a highly regulated step in *Leishmania* purine metabolism.

Abrégé

Les parasites *Leishmania* dépendent de mécanismes de récupération pour acquérir les purines de leur environnement. La GMP réductase (GMPR) catalyse la conversion de GMP en IMP, une réaction cruciale pour maintenir la balance en nucléotide purine. La GMPR de *L. major* (LmGMPR) a été clonée, exprimée et purifiée. L'addition du gène LmGMPR compense pour la déficience en GMPR de souches *E. coli*. L'analyse de la structure quaternaire indique que LmGMPR forme des tétramères et autres complexes d'ordre supérieur en conditions réductrices. Des essais cinétiques ont révélé que l'enzyme ne se comporte pas de façon hyperbolique en ce qui attrait à la GMP mais se conforme à la cinétique de Michaelis-Menten pour le NADPH. L'analyse de la séquence indique que LmGMPR contient des domaines CBS ainsi qu'un site de liaison au MPA. Le MPA compétitionne pour le site de liaison au NADPH avec un K_i de 20μM. l'ATP et le GTP régule l'activité enzymatique via une inhibition et une activation respectivement. Ces donnés indiquent que LmGMPR est une nouvelle enzyme qui exécute une étape hautement régulée du métabolisme des purines de *Leishmania*.

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Contribution of Knowledge to the Field

This Master's research represents truly novel work in the area of enzymology and parasite biochemistry. Here, the first identification of the gene encoding the GMP reductase within the *Leishmania major* genome is described. Preliminary characterization by Spector and Jones (1982) has also been vastly expanded upon. From this information we show that the LmGMPR is a novel GMP reductase in its motif and domain structure. It retains IMPDH-specific characteristics that have never been described before in a GMP reductase. Moreover, the kinetic activity of this enzyme appears to be very complex. It is a highly regulated enzyme suggesting that it plays a critical role in the purine metabolic pathway of *Leishmania.* This information will contribute to our understanding of parasite and enzyme evolution. In addition, this work introduces the LmGMPR as a new potential chemotherapeutic target in the battle against leishmaniasis. The auxotrophic nature of these parasites has made the purine metabolic pathway a desirable target for many researchers throughout the years. Identification of the *Leishmania* GMP reductase may have considerable implications on this field of parasitology. The research conducted in this Master's project has provided a sturdy background for further characterization of this enzyme.

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Introduction

Leishmania species are the causative agents of numerous debilitating diseases known as the leishmaniases. According to the World Health Organization an estimated 12 million people are infected with *Leishmania* world wide and within the 88 countries endemic for the disease there are 350 million people at risk of contracting the parasite. It is estimated that there are 1.5 to 2 million new cases of cutaneous leishmaniasis and 500,000 new cases of visceral leishmaniasis yearly resulting in 2.5 million disability-adjusted life years lost (WHO 2000; WHO 2004).

The disease is caused by parasitic protozoa belonging to the genus *Leishmania.* Upon being transmitted to the host via the bite of an infected sand fly the organism sequesters itself intracellularly. There are a number of clinical manifestations of leishmaniasis depending on the infecting species of parasite. The four most common forms are cutaneous, diffuse cutaneous, mucocutaneous, and visceral leishmaniasis. The disease is primarily zoonotic with canines and rodents serving as reservoirs for the parasite population. However, severe epidemics of leishmaniasis have been associated with anthroponotic spread especially in urban centers (Desjeux 2001).

Leishmania species share the order Kinetoplastida with the causative agents of sleeping sickness and Chagas' disease (*Trypanosoma brucei* and *Trypanosoma cruzi* respectively) due to the presence of the kinetoplast, a unique DNA containing organelle. Members of this order appear to have branched off from the eukaryotic lineage early in

evolution (Dacks et al. 2001). Organisms belonging to the family Trypanosomatidae, within this order, became highly adapted to a parasitic lifestyle (Maslov et al. 2001). These two factors, early divergence and adaptation to parasitism, probably contributed to the divergent metabolic pathways that these parasites retain.

One striking feature is the compartmentalization of specific processes, such as glycolysis, into an organelle called the glycosome (Opperdoes et al. 1977). This organelle also contains many of the enzymes necessary for the salvage of purines (Shih et al. 1998; Jardim et al. 1999; Zarella-Boitz et al. 2004). Since *Leishmania* species are incapable of purine biosynthesis (Marr 1985) the maintenance of this compartment, as well as the enzymes within, is of great interest to researchers.

The purine metabolic pathway of these organisms represents a promising putative drug target. In fact, the drug allopurinol, which exerts its effect on this pathway, has been shown to be selectively lethal to *Leishmania* parasites and has been investigated as a candidate for combination therapy with pentavalent antimony, the first line drug for treating leishmaniasis (Pfaller et al. 1974; Momeni et al. 2002). One mechanism of action of allopurinol is an inhibitory effect on the crucial purine interconversion enzyme guanosine monophosphate (GMP) reductase. GMP reductase is the only known enzyme capable of converting GMP back to the pivotal purine precursor inosine monophosphate (IMP). This step is integral to maintaining a balance in guanylate and adenylate nucleotide pools especially for an organism that relies solely on exogenous purines obtained from the extracellular environment. Spector and Jones (1982) reported partial purification and characterization of the *Leishmania donovani* GMP reductase however the molecular identity of the enzyme was not determined. This study addresses this issue

by identifying the GMP reductase in the *Leishmania major* genome database, cloning and biochemically characterizing the purified enzyme using kinetic and complementation studies.

1. Literature Review

1.1 *Leishmania* species

Leishmania species, along with other Trypanosomatids, are vector borne parasitic protozoans belonging to the Order Kinetoplastida. These parasites have two life cycle stages, the motile extracellular promastigote and the intracellular amastigote, each being morphologically and biochemically distinct organisms (Fig. 1). Promastigotes have an elongated cell body and a single anterior flagellum, while amastigotes lack a flagellar structure and have a rounded appearance (Chang 1985). Within the vector, sandfly genera *Phlebotomus* and *Lutzomyia*, the parasite is primarily in the promastigote form. Upon uptake by the fly, the amastigotes rapidly transform into replicative, non-infective forms referred to as procyclic promastigotes and anchor themselves to the midgut epithelia to prevent expulsion during the digestion process (Pimenta et al. 1994).

The transformation of these forms into metacyclic parasites, the mature infective form, occurs during metacyclogenesis. Studies done with *L. major* and *P. papatasi* indicate that this process involves a lengthening and the subsequent masking of the side chains of the lipophosphoglycan residues important for binding to the midgut (Pimenta et al. 1992). This transformation is thought to enable the parasites to dislodge from the midgut epithelia and travel to the anterior section of the fly. Repositioning within the fly allows transmission to the host during the next feeding. Once in the mammalian host

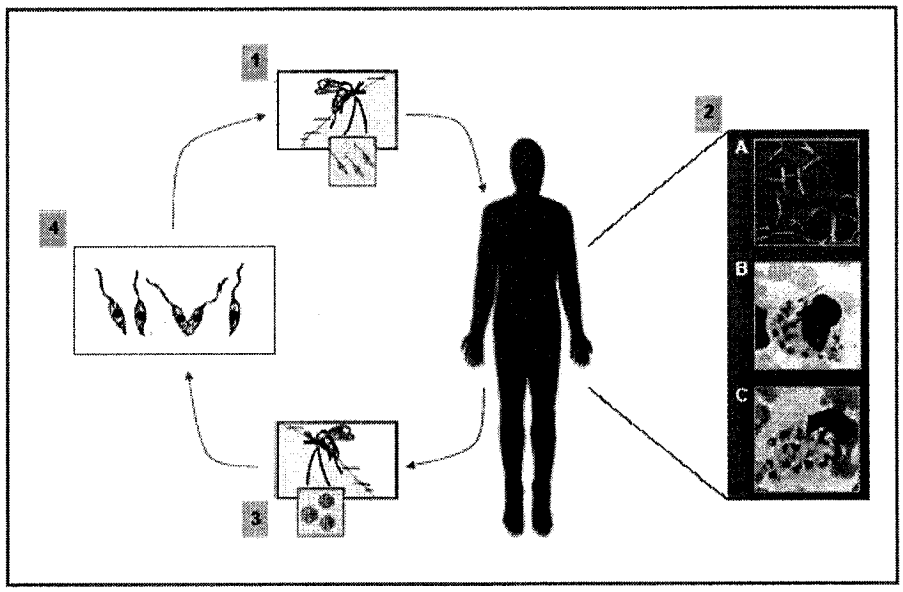

Figure 1. Life cycle of *Leishmania*. 1: *Leishmania* promastigotes enter the host during sand fly feeding. 2: Promastigotes are taken up by macrophages (A) and transform into replicative amastigotes (B). Eventually the macrophage ruptures releasing amastigotes into the surrounding area where they are taken up by new macrophages (C). 3: Amastigotes are ingested during subsequent sand fly feeding. 4: The parasites migrate to the gut of the fly where they transform into promastigotes and replicate.
Adapted from Human Anatomy Online http://www.innerbody.com/htm/body.html; CDC DPDx Image Library http://www.dpd.cdc.gov/dpdx/HTML/Image_Library.htm ; Science Photo Library http://www.sciencephoto.com/

promastigotes are taken up by macrophages through a process of receptor mediated phagocytosis predominantly involving host receptors CR1 and CR3 (Mosser et al. 1985; Rosenthal et al. 1996). Differentiation from the promastigote to the amastigote occurs in the phagolysosomal compartment. Amastigotes continue to proliferate via binary fission, eventually rupturing the host cell. Released amastigotes propagate the infection when they are taken up by new cells. The transmission cycle is completed when a sandfly ingests amastigotes during a blood meal on an infected host (Handman 1999).

Leishmania species have been the subject of much research throughout the years. Most of these studies have utilized the promastigote stage of the parasite. The intracellular locale of amastigotes has made research on their metabolism difficult. As a

result very little information on the biochemical changes that occur between the two stages is available (Looker et al. 1983). Recently, methods for generating axenic amastigotes have been developed by mimicking changes in the host environment with temperature and pH shifts (Pan 1984; Bates 1993; Zilberstein et al. 1994; Gupta et al. 2001). While most alterations are of a quantitative nature (up or down-regulation of metabolic enzymes), adenosine metabolism is quite different between the two stages. Looker and colleagues (1983) showed that conversion of adenosine to hypoxanthine occurs in *L. donovani* amastigotes via an alternate pathway (Fig. 2). An initial cleaving event of adenosine to adenine in promastigotes is replaced by a deamination step

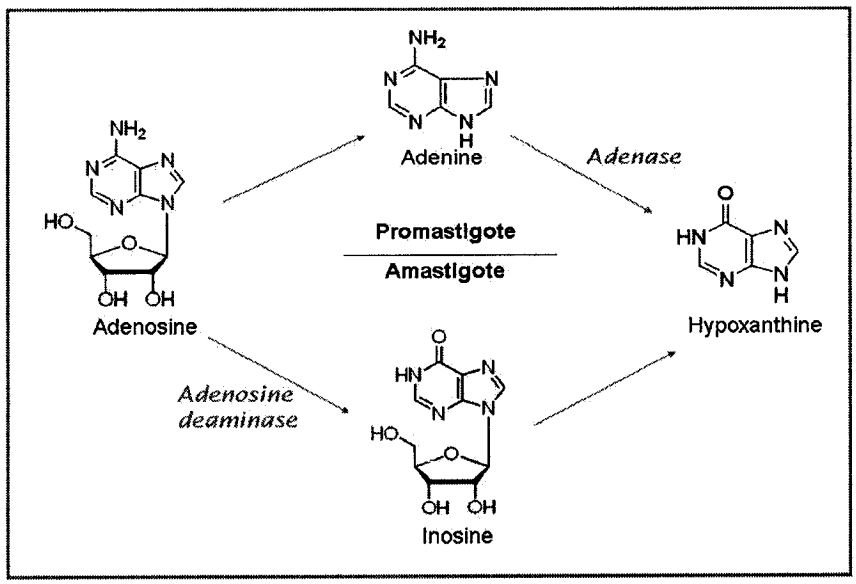

Figure 2. Stage dependent differences in *L. donovani* adenosine metabolism.

catalyzed by adenosine deaminase in amastigotes. The conversion of adenine to hypoxanthine is catalyzed by adenase which appears to be lacking in amastigotes (Konigk et al. 1980). Guanine and xanthine metabolic pathways remain the same in both forms of the parasite.

1.2 Leishmaniasis

The set of diseases caused by *Leishmania* vary depending on infecting species and host response (Convit et al. 1972; Liew et al. 1993). Temperature appears to play an integral role in the site of infection establishment. Species causing cutaneous disease propagate most efficiently at temperatures approximating that of the skin (~35°C) while species that infect visceral organs are more resilient and multiply equally well at 35°C and 37°C (Berman et al. 1981; Zilberstein et al. 1994).

The cutaneous form of the disease, also known as Oriental sore, is most commonly a result of infection with *L. tropica* or *L. major* although there are a number of other *Leishmania* species that can cause cutaneous disease. The infection manifests as a painless ulcerative lesion that normally self heals with the formation of scar tissue. These healed lesions can be aesthetically disfiguring and can be a source of stigmatization for the patient if localized on exposed regions of the body. Resolution of the cutaneous infection confers long-lasting protective immunity (Ashford 2000). Diffuse cutaneous leishmaniasis is a variant of cutaneous disease in which the host immunological response is inadequate in controlling and clearing the infection and is characterized by dissemination of nodules and lesions across the body. The presentation of this form of leishmaniasis leads to disfigurement with common relapses.

Mucocutaneous leishmaniasis, or espundia, is associated with *L. braziliensis* infection. It occurs when the parasite invades the oronasopharyngeal tissues causing destruction and rendering the host susceptible to deadly secondary infections. This form of disease can occur several years after an apparent case of cutaneous leishmaniasis resolves itself. It is primarily a New World phenomenon (Puig et al. 2003).

Visceral leishmaniasis, or kala-azar, is the most severe clinical manifestation of all the leishmaniases. *L. donovani* and *L. infantum*, or *L. chagasi* in the New World, are the species typically associated with this form of leishmaniasis. The disease results when macrophages in the internal organs such as the spleen and liver become infected with the parasite. Symptoms include anemia, fever, and hepatosplenomegaly. Without treatment death is an almost certain outcome of this disease. A variant manifestation of visceral disease called post kala-azar dermal leishmaniasis (PKDL) occurs in patients who have recovered from the initial infection but develop cutaneous nodules that contain large numbers of amastigotes. This form of disease is endemic in Africa and India and not only is the infection a stigmatizing affliction, as the presentation resembles lepratomous leprosy, but infected patients are also potential reservoirs for anthroponotic transmission of the disease (Puig et al. 2003).

1.2.1 Risk Factors

Most cases of leishmaniasis are zoonoses with rodents and canines serving as reservoir hosts (Handman 1999; Ashford 2000) however cases of anthroponotic illness have been reported for cutaneous as well as visceral forms of the infection. Anthroponotic and zoonotic diseases are caused by different species of *Leishmania*. New World zoonotic cutaneous infections are caused by *L. braziliensis* and *L. mexicana* whereas in the Old World this disease is a result of infection with *L. major*. Zoonotic visceral disease manifests upon infection with *L. infantum*. Anthroponotic forms of cutaneous leishmaniasis are caused by *L. tropica* while visceral leishmaniasis is associated with *L. donovani* infection.

Major risk factors for zoonotic illness revolve around movement of populations into areas where the sylvatic reservoirs and the vectors predominate (Desjeux 2001). This includes urbanization, deforestation and housing development, as well as institution of new crops, dam building and irrigation schemes. Initially it was thought that deforestation would decrease the incidence of zoonotic transmission due to loss of habitat for the reservoir and the vector. However, the sprawl of residential zones into forested areas has put more people at risk of coming into contact with the disease not only from the sylvatic reservoir but also from more domesticated reservoirs as the transmission cycle adapts to the new environment. Additionally, the introduction of new crops increases risk for humans because rodents (a common reservoir for *Leishmania*) take advantage of this accessible source of food and consequently nest within or near housing. The ample supply of food also enables the rodent population to multiply thus exacerbating the problem. Dams and new irrigation implementation contribute to the spread of disease by increasing the moisture in the soil and humidity in the air creating a complimentary environment for sand fly survival and propagation (Thakur 2000). Maintenance of the reservoir and the vector populations occurs in many poor suburbs of cities where dogs as well as small garden plots are common (Desjeux 2001).

Anthroponotic illness tends to predominate in crowded conditions and may arise from the migration of populations from rural communities to urban centers. Many factors contribute to the movement of populations some of which are related to socio-economic needs, religious travels, climate (drought and flood) or personal safety (war) (Desjeux 2001). It is of particular concern when migrating populations bring the disease into areas containing non-immune individuals as was the case in the southern Sudanese epidemic in

1984 that led to 100,000 deaths out of approximately 280,000 known inhabitants (Seaman et al. 1996).

The spread of all forms of leishmaniasis increases under a combination of conditions: host specific issues such as malnutrition and immunocompromised status; environmental surroundings that permit survival of the vector such as poor sanitation, proximity of housing to cow sheds, and use of housing materials like mud and dried grass that create favourable habitats for the vector; and socio-economic factors such as lack of treatment resources and health education (Thakur 2000; Desjeux 2001). It is believed that infection with *Leishmania* species only progresses to visceral disease in 1 of every 5 to 10 immunocompetent persons (Alvar et al. 1997). While recent research suggests susceptibility to visceral leishmaniasis infection may have a genetic component (Bucheton et al. 2003) it is still largely a problem for populations living in impoverished conditions where vectors and reservoirs live in close contact with humans.

1.2.2 HIV Infection as a Risk Factor for Leishmaniasis

A divergent, yet concerning, mode of person-to-person transmission of *Leishmania* is emerging. The sharing of needles during intravenous drug use has led to increasing incidence of HIV/leishmaniasis co-infections especially in southern Europe. Intravenous drug users represented 71.1% of the co-infection cases reported and analyzed up to 1998 in this region (WHO 2000). Surveillance systems implemented by the World Health Organization in the mid 1990's have been integral in highlighting the at-risk populations for co-infection however there remain obstacles in estimating these cases (Fig. 3). Surveillance of HIV/leishmaniasis co-infection relies on the hospitals and

physicians to recognize and report cases that may be masked by a combination of diseases. Moreover, leishmaniasis is not included as an "official" opportunistic pathogen associated with HIV infection (Desjeux 1999). Additionally, the lack of adequate surveillance in many African nations where the two infections are endemic results in

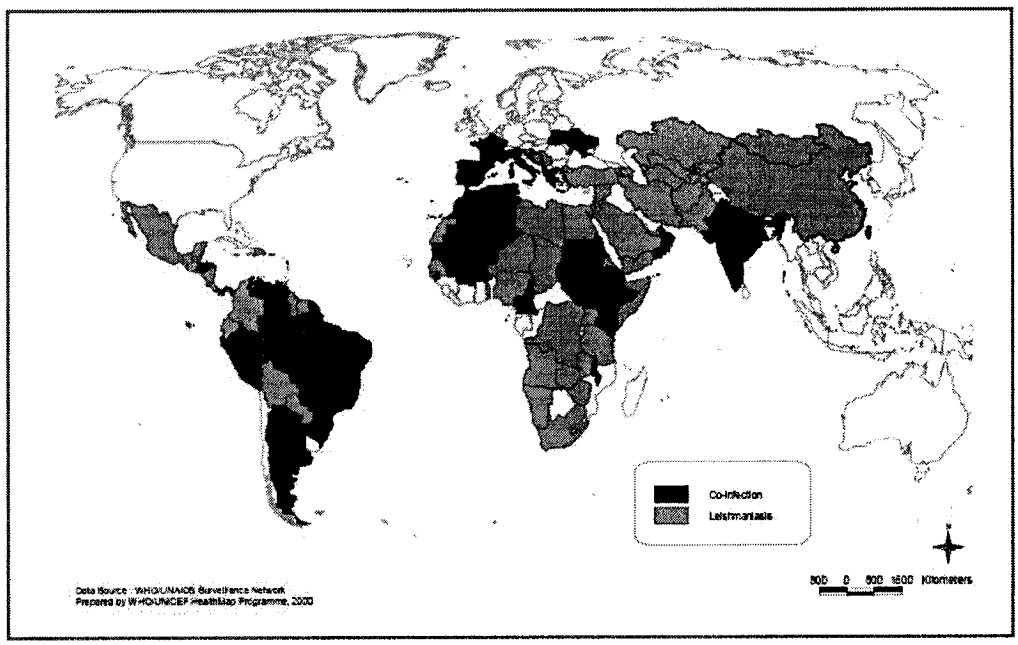

Figure 3. Distribution of Leishmaniasis and HIV/*Leishmania* co-infections. *www.who.int/leishmania/leishmania_maps/en/*

underestimation of co-infection rates (WHO 2000). A major issue in the increase of HIV/leishmaniasis co-infections is the ruralization of HIV spread and the urbanization of leishmaniasis (de Gorgolas et al. 1994). This overlap in transmission area creates an entirely new facet to the spread of both diseases.

Mediterranean countries have seen a drastic change in the presentation of leishmaniasis in recent years. Until 1985, youths under 15 years represented 70% of the leishmaniases cases in Spain. However, by 1997 adult infections predominated with 75% of reported cases. HIV status appears to play a role in this switch of susceptibility (Alvar et al. 1997). HIV infection may induce reactivation of a dormant leishmanial infection or

the viral suppression of the immune system may make progression to full blown leishmaniasis more probable in a primary infection. Due to these effects HIV/*Leishmania* co-infections are often described as being in a vicious cycle of mutual reinforcement.

The type of immune response mounted by the host is a critical determinant of disease progression. The T_h1 response is important for cell mediated immunity and plays a particular role against intracellular pathogens (Janeway 2001). It has been well documented that successful clearance of leishmaniasis requires a T_h1 response while susceptibility is associated with a T_h2 response (Reiner et al. 1995). A T_h1 response has also been suggested to be protective against HIV infection establishment (Clerici et al. 1993). Findings have suggested that HIV may alter cytokine expression driving the immune system towards a humoral response (Chehimi et al. 1994). This not only aids the propagation of HIV within the host but also permits either reactivation or establishment of a primary leishmaniasis infection.

Additionally, the possibility of virus-parasite interactions is relevant due to the ability of both HIV and *Leishmania* to infect the same host cells. Certain variants of HIV have shown tropism to macrophages as well as $CD4^+$ T cells (Mosier et al. 1994). It has also been proposed that parasite lipophosphoglycan can indirectly induce virus expression by stimulating tumor necrosis factor alpha release (Bernier et al. 1995). These synergistic effects on infection coupled with the overlap of endemic areas for both diseases make HIV infection a major risk factor for the contraction of leishmaniasis.

1.2.3 Treatment

Treatment of leishmaniasis is a challenging endeavour. The multiple disease manifestations of leishmaniasis are due in part to the infecting species of parasite. When searching for an anti-leishmanial drug it is important to consider the inherent physiological differences between the species but also between the two morphological stages of the parasite. *Leishmania* species display a preference for different macrophage types which becomes an issue when trying to standardize a course of treatment. Topical preparations have been shown to be effective for dermatological manifestations of disease but a different approach is necessary when targeting parasites that are in the visceral organs. Additionally, the parasites differ biochemically and molecularly across the species which makes finding an effective common target a challenge. There is at least a 3-5 fold variation in sensitivity between species to some of the most common treatments (Croft 2001). The stages of the parasite, promastigote and amastigote, are also biochemically and physiologically distinct: the metabolism of the amastigote is driven by acidic pH (Antoine et al. 1990), metabolic enzyme activities vary between amastigote and promastigote (Coombs et al. 1982), and the protein and glycolipid content of the membrane may differ drastically (Handman et al. 1982; Turco et al. 1991). Targeting drugs to the parasitophorous vacuole housing the parasite is also problematic due to the number of membrane barriers the drug must pass through. Upon administration the compound must pass through the cell membrane of the macrophage, the parasitophorous vacuolar membrane, and finally the membrane of the parasite itself as well as any internal organellar membrane. Additionally, the effect of low pH on drug efficacy and targeting must be considered during development.

Beyond drug efficacy, patient compliance and cost are major concerns as well. Route of administration and length of treatment are two variables that have a significant effect on these two factors. Parenteral administration of anti-leishmanials is common however finding a drug that is taken orally would ease much of the burden associated with treating this disease (WHO 2002; Croft et al. 2006). A number of drugs have been used to treat leishmaniasis however the search for effective, cheap, oral treatments with minimal toxicity is ongoing.

Pentavalent Antimony

The recommended first line treatment for many forms of leishmaniasis are the pentavalent antimonials, sodium stibogluconate and meglumine antimoniate. Courses of treatment with antimonials typically involve up to a month of parenteral administration but they still remain one of the cheapest options in the treatment of leishmaniasis in endemic countries (Berman 1997). Pentavalent antimonials are anti-leishmanial in their trivalent form. It is unknown whether the host macrophage or the parasite reduces the drug to its effective form. Studies have suggested that sodium stibogluconate has an inhibiting effect on glycolysis and β fatty acid oxidation thus affecting parasite metabolism (Berman et al. 1987).

Recent antimonial resistance has emerged in patients suffering from visceral leishmaniasis in Bihar State, India. It is thought that wide spread misuse of pentavalent antimony is applying drug pressure to the parasite and subsequent resistance is arising. In India Sb(V) is available over the counter and as a result many infected individuals do not follow an educated treatment plan (Sundar et al. 1994; Croft et al. 2006). A line of

amastigotes resistant to sodium stibogluconate has been shown to have a decreased capacity to convert Sb(V) to Sb(III) but retain susceptibility to Sb(III) in the medium (Shaked-Mishan et al. 2001). Aquaglycoporin I plays a role in transport of Sb(III). Overexpression of this protein confers enhanced susceptibility to Sb(III) (Gourbal et al. 2004). Antimonial treatment is also problematic for HIV co-infections because this drug requires a T cell immune response to be effective (Murray et al. 1989). Toxic side effects, complex treatment regimens, rising rates of HIV/leishmaniasis co-infections and increasing resistance have led to the push for new anti-leishmanial drugs.

Pentamidine

Pentamidine has been used to treat visceral, cutaneous, and diffuse cutaneous leishmaniasis. The mechanism of action is unknown but it may target kinetoplast DNA (Croft et al. 1982), inhibit polyamine synthesis, and disrupt the mitochondrial inner membrane potential (Vercesi et al. 1992). High parenteral dosing requirements for VL result in increased toxicity and thus pentamidine remains a second line drug for response to antimonial resistance. However, the low doses used to treat CL do not lead to high toxicity and this remains a viable treatment option for this disease (Soto et al. 1994). Resistance is thought to arise from reduced uptake combined with enhanced efflux. Accumulation of the drug in the mitochondrion has been observed and a reduction in this capability may lead to resistance due to the increased availability of the drug for efflux (Basselin et al. 2002).

Amphotericin B

Amphotericin B, an antifungal isolated from *Streptomyces*, has been useful against antimonial resistant and non-responsive infections. The drug binds preferentially to ergosterol moieties in fungi cell membranes. Ergosterol is the predominant sterol of fungi and *Leishmania* plasma membranes. Binding of the drug leads to cell lysis through pore formation in the cell membrane (Brajtburg et al. 1996). Host toxicity effects are due to binding of the drug to cholesterol in mammalian plasma membranes. Amphotericin B has a number of toxic side effects including cardiotoxicity and nephrotoxicity (Croft et al. 2002). Fortunately, lipid associated constructions decrease the risk of toxicity and increase the plasma half life.

These formulations are not in wide use in developing countries due to high cost however they are very effective against antimony resistant visceral leishmaniasis and remain a viable option for serious cases (Croft et al. 2002; Croft et al. 2006). Resistance mechanisms against this drug involve an alteration in membrane structure. A laboratory strain of *L. donovani* promastigotes selected for resistance to amphotericin B showed a switch from utilization of ergosterol as the primary membrane sterol to its precursor cholesta-5,7,24-trien-3β-ol (Mbongo et al. 1998).

Paromomycin

Paromomycin is an anti-parasitic agent that has been used successfully against intestinal amebiasis (Pamba et al. 1990) and *Cryptosporidium* (Bissuel et al. 1994). It is an aminoglycoside that interferes with bacterial protein synthesis. This drug has been used both topically and parenterally. Specific ointment formulations containing 15%

paromomycin were highly effective against *L. major* cutaneous lesions on BALB/c mice (El-On et al. 1984) and subsequently marketed after succeeding in human trials (El-On et al. 1992). Differential topical formulations have shown variable efficacy depending on the infecting species of *Leishmania* (Croft et al. 2002).

To treat visceral forms of the disease the drug must diffuse systemically. Research on the efficacy of parenteral administration of paromomycin has been conducted and clinical studies have shown high cure rates and minimal side effects with this treatment (Jha et al. 1998). It is unclear what the mechanism of action is with this drug although there is evidence that it may cause perturbation of mitochondrial ribosomes and the mitochondrial membrane potential (Croft et al. 2006). Resistance has been associated with decreased uptake in *L. donovani* promastigotes (Maarouf et al. 1998).

Miltefosine

Originally developed as anti-tumor agents (Runge et al. 1980), a promising new class of drugs in the treatment of leishmaniasis are the alkyllysophospholipids (Croft et al. 2002). The most notable amongst them is miltefosine. This compound has the capacity to be delivered orally which is a distinct benefit over current parenteral treatments. Unfortunately, the mechanism of action of these drugs remains unknown. A suggested target in *L. donovani* promastigotes are the lipid biosynthetic enzymes (Achterberg et al. 1987). Miltefosine has shown high efficacy against *L. donovani* in mouse models (Kuhlencord et al. 1992). Used as an oral treatment in Bihar, India it had a 95% cure rate for mild to moderate cases of visceral leishmaniasis (Sundar et al. 1998; Jha et al. 1999). Additionally, studies done using immunodeficient mice indicate that

miltefosine may also be effective in treating HIV/*Leishmania* co-infections (Murray et al. 2000). However, miltefosine's teratogenicity precludes its use in women of child bearing age.

1.3 Purine Metabolism

Purine nucleoside mono/di/tri-phosphates are critical for cellular energy storage and DNA synthesis. While humans utilize *de novo* synthesis and salvage pathways, some divergent organisms, such as kinetoplastids and the *Mycoplasma* bacteria, completely lack the genes required for *de novo* synthesis (Berens 1995). The reason for lack of a purine biosynthetic route in protozoans remains unclear. Possibly these divergent organisms never developed the ability to synthesize purines (Hitchings 1982) or there may have been multiple events in which the genes were individually slowly lost over time (Becerra et al. 1998). The absence of a biosynthetic route in *Leishmania* amplifies the necessity of the enzymes involved in salvage and in the interconversion of the purine bases. While this is a biochemically interesting feature of these parasites it also has important implications for drug target development.

1.3.1 Purine Biosynthesis

In humans the liver is the main site of purine biosynthesis. Guanine and adenine purine synthesis occurs sequentially as the purine ring is assembled on a phosphorylated ribose (Nelson 2005). Completion of the purine ring results in the formation of a generic nucleotide called inosine monophosphate (IMP). This is followed by conversion of IMP to either GMP or AMP. The initial step of purine synthesis involves amination of

5'phosphoribosyl-1-pyrophosphate (PRPP) via transfer of an amine group from glutamine. Glycine is then joined to the ribose via the new amine group and the peptide hydroxyl group. This creates half of the purine base ring. Subsequent steps build the rest of the ring in a stepwise fashion. Fusion of aspartate to the ring initiates the structural assembly of the second ring. The closure of the ring forms the purine inosinate, or IMP. IMP is the pivotal precursor to the formation of both AMP and GMP (Fig. 4). AMP is formed via two consecutive reactions involving the enzymes adenylosuccinate synthetase

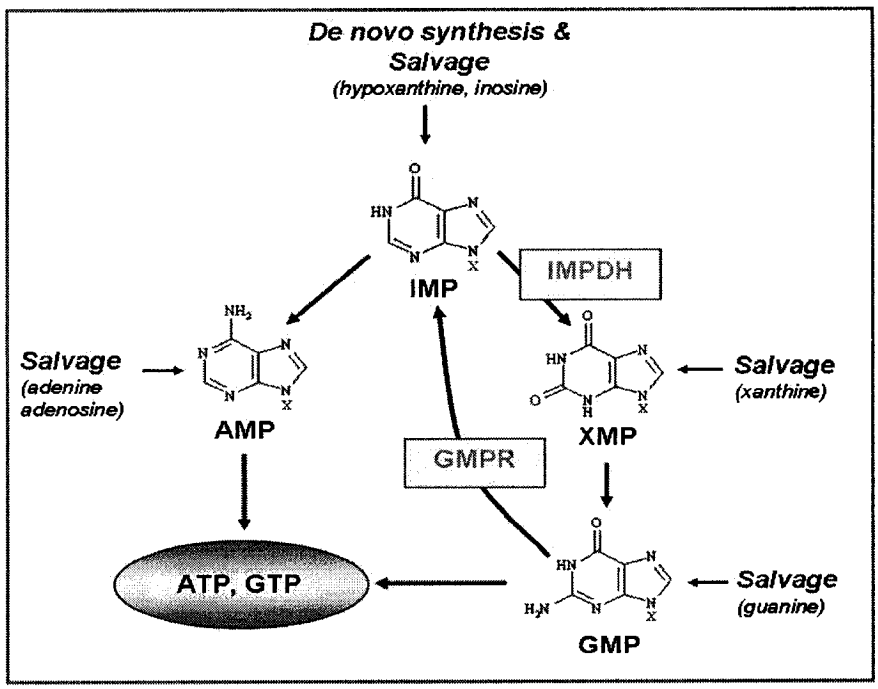

Figure 4: Enzymatic reactions that generate GMP and AMP. Inosinate is the precursor to the formation of both GMP and AMP. IMPDH converts IMP to XMP in the first step of the formation of GMP. GMP can be brought back to IMP in one step catalyzed by GMP reductase. Not shown here, AMP can also be converted back to IMP via adenosine deaminase. Salvageable purine components are bracketed at the step in which they enter the pathway.

and adenylosuccinate lyase which use aspartate to aminate IMP to AMP releasing fumarate. GMP is formed from IMP via the action of IMP dehydrogenase, which inserts

an oxygen atom at C2 of the ring, and XMP glutamine amidotransferase, which replaces the new oxygen atom with an amine group from glutamine. Typically, AMP synthesis is driven by GTP and the formation of GMP is ATP dependent. This may serve a regulatory function (Nelson 2005). Subsequent steps result in formation of nucleoside triphosphates and either retention as energy carriers or incorporation into nucleic acids. Integral to this pathway is the ability to interconvert purines. GMP reductase catalyzes the reaction of GMP back to IMP while AMP is converted to IMP via AMP deaminase.

1.3.2 Mechanisms of Human and Leishmania Purine Salvage

Nucleotide biosynthesis is supplemented in many organisms by the presence of salvage mechanisms. Purine salvage pathways play an important role in mediating the balance of purine nucleotides and the recycling of previously synthesized nucleotides. *Leishmania* species utilize both pyrimidine biosynthesis and salvage mechanisms however these organisms do not contain a biosynthetic route for purines and as a result rely exclusively on purine salvage for survival (Marr et al. 1978; Marr 1985)

Import of purines and their corresponding nucleosides varies between *Leishmania* species. *L .donovani* has been shown to have two purine nucleoside transporters, LdNT1 specific for adenosine and pyrimidines and LdNT2 specific for inosine and guanosine (Aronow et al. 1987). *L. braziliensis panamensis* appears to have three transporters (Hansen et al. 1982). One is specific for the nucleobases adenine and hypoxanthine, the second selectively imports inosine, and the third adenosine. The K_m values for LdNT1 and LdNT2 are two orders of magnitude lower than those of mammalian nucleoside transporters (Plagemann et al. 1981; Plagemann et al. 1985; Cohn et al. 1997). This

characteristic would be beneficial during the intracellular stage when the parasite may need to out-compete the host for the available exogenous purines.

Upon entering the cell, purine nucleosides and nucleobases can be shunted into one of two salvage pathways. The first involves enzymes called phosphoribosyltransferases (PRTs) that catalyze the single step addition of a specific purine base to PRPP to form the corresponding nucleoside monophosphate:

$$Base + PP\text{-}ribose\text{-}P \rightarrow base\text{-}ribose\text{-}P + PP_i$$

The second pathway involves the dephosphorylation of ribose-1-phosphate and simultaneous attachment of the purine base, catalyzed by a phosphorylase, followed by phosphorylation of the nucleoside by a nucleoside kinase (Glew et al. 1988):

$$Base + ribose\text{-}1\text{-}P \rightarrow base\text{-}ribose + P_i$$

$$Base\text{-}ribose + ATP \rightarrow base\text{-}ribose\text{-}P + ADP$$

High levels of phosphoribosyltransferase activity have led to suggestions that *Leishmania* and *Trypanosoma* preferentially scavenge purine bases (Davies et al. 1983; Glew et al. 1988). The detection of 3' nucleotidase activity at the cell surface of *Leishmania donovani* as well as the presence of nucleoside transporters (Hansen et al. 1982; Dwyer et al. 1984) indicate that nucleoside scavenging may also be of great importance to fulfill the parasite's purine requirement. The nucleoside kinase activity in *L. donovani* (Datta et al. 1987) has led to the suggestion that exogenous nucleic acids and nucleotides are

salvaged from the host by dephosphorylation at the membrane and transported across the membrane as nucleosides (Gottlieb 1989). Within the parasite, nucleosides are either rephosphorylated by a nucleoside kinase or broken into ribose and purine components and subsequently converted to a nucleotide level by phosphoribosyltransferases.

Three PRTs have been identified in *Leishmania* species. Two of these, hypoxanthine-guanine PRT (Shih et al. 1998) and xanthine PRT (Jardim et al. 1999; Zarella-Boitz et al. 2004), have been localized to the glycosome whereas the third, adenine PRT, has been shown to be cytosolic (Zarella-Boitz et al. 2004). The glycosomal localization of purine salvage enzymes specific for hypoxanthine, guanine, and xanthine indicates that the guanine portion of purine production may occur in this compartment. This speculation is further supported by the finding that the IMPDH responsible for converting IMP to GMP is also a glycosomal enzyme (Jardim, A. personal communication). The portion of the purine biosynthetic pathway involved in conversion of IMP to AMP and GMP is intact in *Leishmania* (Glew et al. 1988) however the precise subcellular location of many of the enzymes in this pathway has not been established.

1.3.3 Glycosomal Localization

The glycosome is a divergent organelle evolutionarily related to peroxisomes and glyoxysomes. Specific to Trypanosomatids, it compartments a large portion of the glycolytic pathway (Opperdoes et al. 1977), ether lipid biosynthesis (Opperdoes 1984), β-oxidation of fatty acids (Wiemer et al. 1996), and a number of purine salvage enzymes (Shih et al. 1998; Jardim et al. 1999; Zarella-Boitz et al. 2004). Glycosomal enzymes are targeted to the organelle through an organized import mechanism. Two cytosolic

receptors, PEX5 and PEX7, guide proteins bearing a **P**eroxisomal **T**argeting **S**ignal (PTS) sequence-1 and 2, respectively to the glycosome for importation (Gould et al. 1989; Swinkels et al. 1991). The typical PTS-1 is (S/A/C)-(K/R/H)-L located at the C-terminal end of the protein to be imported. In contrast, PTS-2 sequences are near the N-terminus and consist of (R/K)(L/V/I)X_5(Q/H)(L/A). The localization of purine salvage enzymes to this compartment may represent an interesting aspect of purine metabolism in these organisms. The purpose of this compartmentation is unknown.

1.4 GMP Reductase

In 1960 Mager and Magasnik showed the presence of an enzyme capable of interconverting purines in *S. typhimurium*, *A. aerogenes*, and *E. coli* (Mager et al. 1960). Enzymatic analysis showed this activity to be that of GMP reductase, which catalyzed a single deamination of GMP to IMP in an irreversible manner. GMP reductase activity was also demonstrated in mammalian cells such as calf thymus (Stephens et al. 1973) and human erythrocytes (Mackenzie et al. 1973). Studies on the crustacean *Artemia salina* GMP reductase indicated that the enzyme was inhibited by XMP and activated by GTP (Renart et al. 1976). Thus in the presence of high levels of guanylate nucleotide pools GMP reductase could be stimulated to start converting excess guanylate to adenylate. Subsequently, Spector et al. (1979) used this information to investigate the characteristics of human GMP reductase (Spector et al. 1979). Bimodal substrate saturation curves, similar to *A. salina*, suggested that the human enzyme contained either two different enzymes that catalyzed the same reaction or the enzyme consisted of subunits that acted in a negatively cooperative fashion (Levitzki et al. 1969). Recently it has been shown

that there are indeed isoenzymes of human GMP reductase (Deng et al. 2002; Zhang et al. 2003).

Distribution of GMP reductases appears to be variable across phylogenetically diverse organisms. It is present in *E. coli* (Mager et al. 1960), humans (Mackenzie et al. 1973), and the crustacean *Artemia salina* (Renart et al. 1976) but has not been found in *Drosophila* or *H. influenzae* (Becker 1974; Fleischmann et al. 1995; Becerra et al. 1998). Until recently rodents were thought to lack this enzyme (Kanno et al. 1989) but Salvatore et al. (1998) have identified a rat protein that shares ~95% amino acid identity with the human GMP reductase (Salvatore et al. 1998). However, this group failed to substantiate their claim with kinetic and biochemical data. Previously, GMP reductase activity had been demonstrated in crude extracts of *L. donovani* promastigotes (Spector et al. 1982) but the gene encoding this protein has remained unidentified until this study.

1.4.1 GMP reductase/IMPDH Structure

GMP reductases and IMP dehydrogenases belong to a family of enzymes characterized by the presence of a TIM barrel structure (Zhang et al. 1999). It has been estimated that 10% of all enzymes belong to this structural family while retaining very different functions (Reardon et al. 1995). The TIM structure contains α helices and β sheets complexed together to form a barrel. Although there is usually little sequence homology between the members of this family they all retain their active site at their C terminus (Farber et al. 1990). Both IMPDH and GMP reductase have been shown by motif searches to have a conserved phosphate binding site near their C terminus (Bork et al. 1995). The sequence identity and similarity between *E. coli* IMPDH and GMP

reductase is 36% and 52% respectively. Due to the substantial sequence similarity, misclassification of GMP reductase as an IMPDH is plausible especially if homology is the only determinant used.

There are three sequence motifs that are typically associated with IMPDH enzymes; an IMPDH/GMP reductase signature motif, two tandem **C**ystathionine **B**eta **S**ynthase (CBS) domains, and a mycophenolic acid (MPA) binding site. Both GMP reductases and IMPDH enzymes contain the IMPDH/GMP reductase signature. The only IMPDH shown thus far to not contain CBS domains is from *Borrelia burgdorferi* (Zhou et al. 1997). However, to date no GMP reductases have been shown to contain CBS domains or MPA binding sites.

Within the IMPDH/GMP reductase signature motif is an IMP binding site thought to involve a crucial cysteine residue (Andrews et al. 1988), sequence **GS(I/V)C(I/T)T**, with the amino acid substitutions occurring in GMP reductases. Both enzymes catalyze reactions involving IMP and nicotinic dinucleotide cofactors (Digits et al. 1999). GMP reductases catalyze the single step deamination of GMP via the reaction:

$$GMP + NADPH + H^{+} \leftrightarrow IMP + NADP^{+} + NH_3$$

While IMP dehydrogenases catalyze the reaction:

$$IMP + NAD^{+} + H_2O \leftrightarrow XMP + NADH + H^{+}$$

These similarities in substrate specificities contribute to the homology of the two enzymes.

The second characteristic IMPDH motifs are the CBS domains. These were first identified as important conserved structures in 1997 in the enzyme cystathionine β synthase (Bateman 1997). Before this discovery it was noted that the *E. coli* K12

IMPDH, encoded by the *guaB* gene, had an insert of 123 amino acids that was lacking in the K12 GMP reductase gene, *guaC* (Andrews et al. 1988). It had been suggested that in the evolution of these two enzymes the additional amino acids were either inserted into *guaB* or they were lost from *guaC* (Andrews et al. 1988). This insert corresponds to two tandem CBS domains. CBS domains have been associated with a regulatory role, binding adenosine derivatives (Scott et al. 2004), and intracellular trafficking (Schmidt-Rose et al. 1997). The crystal structure of the *Streptococcus pyogenes* IMPDH indicates that the CBS domains of this enzyme protrude from the main catalytic domain (Zhang et al. 1999) where they may serve as binding sites for regulatory molecules.

In the human IMPDH 1 enzyme the amino acid substitution D226N in the second CBS domain causes the autosomal dominant form of the disease retinitis pigmentosa (Bowne et al. 2002). However, a deletion of the entire CBS domain of human IMPDH II had no effect on catalytic activity (Sintchak et al. 1996) and the *B. burgdorferi* IMPDH, lacking CBS domains, retains similar activity to other IMPDH enzymes (Zhou et al. 1997). Unfortunately, neither of the two studies addressed the issue of a regulatory role for the CBS domains. Catalytic activity may not be affected with the absence of CBS domains if their primary function is regulation.

The third characteristic associated with IMPDH enzymes is the MPA binding site. Mycophenolic acid (MPA) is considered an IMPDH-specific inhibitor. Its mechanism of action on IMPDH has been extensively characterized (Fig. 5) (Sintchak et al. 1996; Digits et al. 1999; Gan et al. 2002). MPA binds the dinucleotide site of the enzyme after release of NADH effectively trapping the XMP intermediate. The conformational change required to hydrolyze the intermediate is physically blocked by the presence of the

inhibitor. Recently, it has been shown that the effect of MPA inhibition is species-specific. Microbial IMPDHs tend to be resistant to MPA inhibition while mammalian

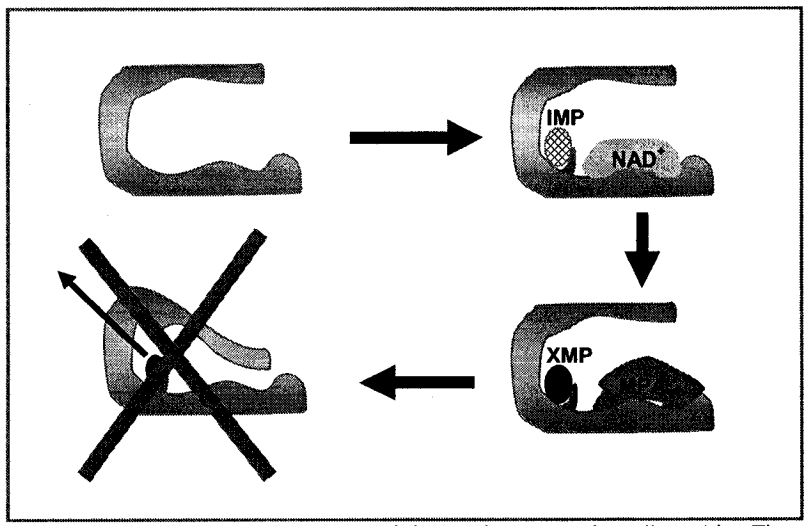

Figure 5. Mechanism of IMPDH inhibition by mycophenolic acid. The enzyme binds NAD^+ and IMP. NADH is released after hydride abstraction from IMP. MPA binds the dinucleotide binding site prohibiting the enzyme flap from hydrolyzing the E-XMP intermediate. *Adapted from (Umejiego et al. 2004)*

IMPDHs are sensitive (Verham et al. 1987; Carr et al. 1993; Sintchak et al. 1996; Zhou et al. 1997). This appears to be due to the existence of two different amino acid sequences associated with binding MPA. Mammalian IMPDHs have the sequence **AQGVSG**, that corresponds to a K_i of 10-20 nM while microbial enzymes, sequence **PEG(I/V)EG**, have a K_i of 10-20 μM.

Due to cellular requirements for guanosine derivatives, IMPDH plays a pivotal role in cell proliferation and differentiation (Jackson et al. 1975; Collart et al. 1990). Consequently, the inhibition of IMPDH by MPA has immunosuppressive, anti-viral, and anti-tumor effects. A form of MPA, marketed as CellCept, is in clinical use to prevent graft rejection for organ transplants (Mele et al. 2000).

1.4.2 The Leishmania GMP Reductase

GMP reductases catalyze a biologically significant reaction in cells. *Leishmania donovani* relies heavily on the ability to scavenge purines and nucleosides from the host organism and must be efficient at incorporating these exogenous molecules into their metabolic pathways. Since GMP reductase is the only enzyme known thus far that catalyzes the conversion of GMP to IMP it may play an invaluable role in the biochemistry of *Leishmania* species. Prior work by Spector and Jones (1982) showed that there did appear to be an enzyme exhibiting GMP reductase activity in *L. donovani* and the presence of an uncharacterized, IMPDH-like gene in the *L. major* genome gives credence to the hypothesis that *Leishmania* species utilize a GMP reductase in their purine metabolic pathway. This work has approached the hypothesis from a genomic standpoint. Cloning of the putative *GMPR* gene from the *L. donovani* genome into various vectors has enabled the expression and purification of the enzyme. Kinetic studies and complementation of function in auxotrophic *E. coli* cell lines has been used to characterize the enzymatic reaction. Due to the substantial sequence similarity between the *Leishmania* GMP reductase and the IMPDH as well as the retention of two IMPDH-specific characteristics, CBS domains and the MPA binding site, vestigial IMPDH activity and the role of these two sites has been investigated.

1.5 Conclusion

GMP reductases are the only known catalysts that convert GMP back to IMP. This is an essential activity in organisms that rely on the environment to supply their purines as this enzyme provides a mechanism for ensuring a homeostatic balance in the

guanine and adenine pools within the cell. Although GMP reductase activity in *L. donovani* has been partially characterized (Spector & Jones 1982) little is known about its genomic organization, subcellular localization, or whether this enzyme is critical for parasite viability. BLAST searches of the *L. major* genome database have revealed two genes encoding IMPDH's however no GMP reductase gene is proposed. While one gene has been biochemically characterized the other remains classified as an IMPDH simply based on homology. Clustal W multiple sequence alignments have shown that these proteins share 54% positive identity that could result in misidentification (Chenna et al. 2003). The similarities between the two types of enzymes suggest that the gene lacking characterization codes for a GMP reductase. This is supported by Spector and Jones' (1982) work, the essential nature of purine salvage and lack of *de novo* biosynthesis in *Leishmania* species, as well as the lack of evidence thus far for any other enzyme(s) that may perform a similar function to that of the GMP reductase.

2. Materials and Methods

2.1 Chemicals and reagents

All restriction endonucleases and buffers used for cloning were purchased from Invitrogen. Alamar Blue was obtained from Medicorp. Mycophenolic acid was purchased from MP Biomedicals. GTP and GMP cross-linked agarose beads were obtained from Sigma. All other reagents were of the highest quality commercially available.

2.2 Cloning and Expression of LmGMPR

The *GMPR* open reading frame was amplified by polymerase chain reaction from *L. major* genomic DNA with the sense, 5'-CATGCCATGGCAGCCCTAGGCAGTC-3', and antisense, 5'-GGAATTCTTACAGCTTCGAGATATCGTG-3', primers containing *NcoI* and *EcoRI* restriction endonuclease sites (underlined), respectively, using *Pfx* DNA polymerase (Invitrogen) and 30 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s, and extension at 72 °C for 90 s. The *LmGMPR* ORF was cloned into the *NcoI* and *EcoRI* sites of the pET30-b(+) and the pBAD expression vectors. The integrity of these constructs was confirmed by DNA sequence analysis.

E. coli strain *ER2566* was transformed with pET30-b(+)-*LmGMPR*. For protein expression, *E. coli ER2566* pET30b(+)-*LmGMPR* cells were cultured in Luria Broth (LB) containing 50 μg/mL kanamycin at 37°C to an OD_{600} of 0.7. Induction was initiated by adding isopropyl-beta-D-thiogalactopyranoside (IPTG) to a final concentration of 0.7 mM and was carried out at 20°C with shaking for 3 h. Bacterial cultures were harvested by centrifugation and resuspended in 20 mL of 20 mM Tris pH 8. Cells were lysed with three passes through a French press and the lysate was clarified by centrifugation at 14,000 rpm for 20 min at 4°C. The supernatant was loaded on a Ni^{2+} nitrilotriacetic acid bead column 1.5 X 12 cm pre-equilibrated with TBS (40 mM Tris pH 8.0 and 0.5 M NaCl). Unbound proteins were removed by washing the column with 1 column volume of 0.4 M NaCl in TBS. A step gradient of 10, 20, 40, 80, 160, and 320 mM imidazole (15 mL/step) in TBS were used to elute the His_6/S-LmGMPR enzyme. Samples from each fraction were run on SDS-PAGE and stained with Coomassie Blue R-250.

Fractions containing a homogeneous 50 kDa protein were concentrated in a 15 mL KMWCO Millipore centrifugal concentration filter. During the concentration step the TBS imidazole solution was replaced with PBS (100 mM potassium phosphate buffer, 150 mM NaCl pH 8). Purified enzyme was quantified at 280 nm with a Beckman spectrophotometer by the method of Gill and von Hippel using an ε_{280nm} of 9970 $M^{-1}cm^{-1}$ (Gill et al. 1989). The enzyme was stored at 4°C in PBS with 1 mM dithiothreitol (DTT) and 1 mM EDTA.

For expression of untagged LmGMPR the open reading frame was cloned into the pBAce plasmid via sticky/blunt ligation. The construct pET30-b(+)-*LmGMPR* and the empty pBAce plasmid were digested for 3 h with *EcoRI* and *EcoRV*, respectively. These sites were blunted with T4 DNA polymerase for 30 min at 20°C, precipitated, and digested with *NcoI* for 12h at 37°C. The fragments were gel purified from an agarose gel using the Qiagen gel purification kit and ligated with T4 DNA ligase at 20°C for 12h. The pBAce-LmGMPR construct was transformed into a GMP reductase deficient cell line, *E. coli* K12 Δ *gua C* strain H1174.

In pBAce, LmGMPR expression is under the control of the leaky *phoA* promoter which is regulated by low phosphate conditions in the culture media (Craig et al. 1991). Protein expression is induced under conditions of depleted phosphate. Growth of the pBAce-*LmGMPR* Δ *gua C* culture at 37°C for 24 h in LB media with 50 μg/mL of streptomycin sulfate and 100 μg/mL ampicillin resulted in efficient induction of the enzyme. Cells were harvested by centrifugation and resuspended in 50 mM Tris pH 7.6 with 2 mM EDTA and 10 mM β-mercaptoethanol (TEM) and lysed as described earlier. Clarified lysates were sequentially saturated to 25 and 50% with ammonium sulfate. The

50% protein pellet was resuspended in 20 mL of TEM and loaded onto a 1.5 X 2 cm Cibacron Blue-Sepharose (Pharmacia) column. The column was washed with 5 column volumes of 0.3 M KCl in TEM. LmGMPR was eluted with a step gradient of 0.4 M to 1 M KCl in TEM. These fractions were pooled, the buffer was exchanged for PBS and concentrated using a 15 mL KMWCO Millipore centrifugal concentration filter. The enzyme was stored in PBS with 1 mM DTT and EDTA on ice at 4°C.

2.3 Complementation

Gua B, Δ *IMPDH*, and Gua C, Δ *GMPR*, deficient K12 *E. coli* mutants, strains H724 and H1174 respectively, were obtained from the Yale *E. coli* Stock Center and transformed with pBAD-*LmGMPR*. These mutant *E. coli* cells also lack the enzymes necessary for *de novo* purine biosynthesis. The pBAD-*LmGMPR* product lacks a tag and is inducible with 0.2 % L-arabinose. Transformants were grown on Luria Broth (LB) plates containing 50 μg/mL ampicillin and 50 μg/mL streptomycin sulfate. A colony from the LB ampicillin/ streptomycin plates was suspended in 100 μL of modified arabinose induction media and streaked out on MAI 3 % agarose plates supplemented with 100 μM tyrosine, tryptophan, proline, glutamine, threonine, and histidine, 50 μg/mL ampicillin, and 50 μg/mL streptomycin sulfate, and either 50 μg/mL hypoxanthine, 35 μg/mL adenine, 35 μg/mL guanine, all three of these purines (positive control) or no purines (negative control). The MAI media contains 40 mM MOPS, 4 mM Tricine, 0.01 mM $FeSO_4$, 9.5 mM NH_4Cl, 0.28 mM K_2SO_4, 0.5 μM $CaCl_2$, 0.53 mM $MgCl_2$, 50 mM NaCl, 0.2 % casamino acids, and 10 μL of a micronutrient solution (3 nM $(NH_4)_6(MO_7)$; 400 nM H_3BO_3; 30 nM $CoCl_2$; 10 nM $CuSO_4$; 80 nM $MnCl_2$; 10 nM $ZnSO_4$), 1.32 mM

K_2HPO_4, 0.2 % arabinose, 5 μg/mL nicotinic acid, and 5 μg/mL thiamine. As a control for bacterial viability, LB plates containing only streptomycin were streaked with the two parental strains Δ *guaB* and Δ *guaC*, and the plasmid control and the pBAD-*LmGMPR* construct in each strain.

The construct pBAce-*LdIMPDH* was used to complement IMPDH activity in Δ *guaB E. coli* cells. Transformants were generated as discussed earlier and a colony from each of the LB ampicillin/ streptomycin plates was suspended in 100 μL of modified low phosphate induction (LPI) media and streaked out on LPI agarose plates containing 100 μM supplemental amino acids tyrosine, tryptophan, proline, glutamine, threonine, and histidine, 50 μg/mL streptomycin sulfate, and 50 μg/mL ampicillin. The plates were then supplemented with either 50 μg/mL hypoxanthine, 35 μg/mL adenine, 35 μg/mL guanine, all three of these purines (positive control) or no purines (negative control). The LPI media contains 40 mM MOPS, 4 mM Tricine, 0.01 mM $FeSO_4$, 9.5 mM NH_4Cl, 0.28 mM K_2SO_4, 0.5 μM $CaCl_2$, 0.53 mM $MgCl_2$, 50 mM NaCl, 0.2% glucose, 0.2% casamino acids, 1.5 μM thiamine, and 10 μL of a micronutrient solution (3 nM $(NH_4)_6(MO_7)$; 400 nM H_3BO_3; 30 nM $CoCl_2$; 10 nM $CuSO_4$; 80 nM $MnCl_2$; 10 nM $ZnSO_4$). As a control for bacterial viability LB plates containing only streptomycin (LBS) were streaked with the two parental strains Δ *guaB* and Δ *guaC* and the plasmid control construct, pBAce-*LdIMPDH*, in each strain.

For a quantitative assessment of complementation, the same experiment was performed with the liquid form of each media. Cultures of each of the cell lines were grown in liquid MAI or LPI media for 24h at 37°C with shaking. Growth was measured

by recording the OD_{600} for each. This experiment was performed in triplicate. Significance was determined via the Student's T-test.

2.4 Enzyme Kinetics

Standard His_6/S-LmGMPR assays were performed in 75 mM sodium phosphate buffer pH 6.9, 1 mM DTT, 1 mM EDTA (Buffer A) with 185 nM LmGMPR at 30 °C. Oxidation of NADPH at 340 nm (ε= 6200 $M^{-1}cm^{-1}$) was measured on a Beckman-Coulter DU640 spectrophotometer. Determination of K_m values for the substrates GMP and NADPH was performed by individually varying the concentration of GMP (5-500 μM) at a fixed NADPH concentration of 45 μM, and by varying the concentration of NADPH (5-200 μM) at a fixed GMP concentration of 100 μM. Initial rate data was analyzed by direct linear plots (Eisenthal et al. 1974) and fit to the Michaelis-Menten equation. For convenience the data is presented in the more conventional Hanes-Woolf plot. The reverse reaction was measured at varying concentrations of IMP (20-100 μM), 100 μM ammonium chloride or 200 μM ammonium acetate, 45 or 100 μM $NADP^+$ and 371 or 927 nM LmGMPR. IMPDH reactions were assessed over 5-200 μM IMP, and 10-200 μM NAD^+ or $NADP^+$ in 50 mM Tris pH 7.5, 100 mM KCl, 1 mM DTT, and 1 mM EDTA at 30 °C.

For untagged LmGMPR, assays were performed using 46 nM enzyme concentrations (2.4 μg of enzyme/reaction) in the presence of varying concentrations of GMP (20-500 μM) at a fixed NADPH concentration of 100 μM and with varying concentrations of NADPH (5-100 μM) at a fixed GMP concentration of 300 μM. Effector compounds, such as ATP and GTP, were used over the range of 5 μM – 1mM.

The assay buffer was sodium phosphate buffer pH 6.9 containing 1mM DTT, 1mM EDTA, and 100 mM KCl.

N-ethylmaleimide (NEM), diethyl pyrocarbonate (DEPC), and diacetyl were used to elucidate crucial amino acid residues. His_6/S-LmGMPR was incubated with 5 μM NEM for increasing time increments (0, 1, 5, 10 min). Kinetic assays were conducted with 45 μM NADPH, 100 μM GMP, and 278 nM LmGMPR in 75 mM sodium phosphate buffer pH 6.9 with 1mM DTT and EDTA. Diacetyl (1 mM) was incubated with the tagged enzyme for up to 30 min and kinetic activity was assessed as mentioned above. DEPC (1, 5, and 10 mM) was incubated with the untagged form of the enzyme for 15 min. Kinetic assays were conducted with 100 μM NADPH, 300 μM GMP, and 46 nM LmGMPR in 75 mM sodium phosphate buffer pH 6.9 with 1mM DTT and EDTA.

2.5 Mycophenolic acid inhibition kinetics

MPA inhibition of LmGMPR was done using a range of MPA concentrations (0-100 μM) with GMP fixed at 100 μM and varying NADPH concentrations or under a fixed concentration of NADPH (45 μM) and varying GMP. All reactions were performed using standard LmGMPR assay conditions. Data was fit to the Hanes-Woolf linear transformation and the type of inhibition interpreted from these graphs. The K_i for MPA was obtained by replotting the K_{mapp}/velocity for each concentration of MPA from 0-50 μM versus MPA concentration (Kakkar et al. 1999).

2.6 Sucrose Gradients

Preparation of 5-20% sucrose gradients was done by overlaying 1.7 mL of sucrose stock solutions (20, 17.5, 15, 12.5, 10, 7.5, and 5%) individually prepared in 75 mM sodium phosphate buffer pH 6.9 with 5 mM β-mercaptoethanol and 1 mM DTT (buffer A). The gradient was linearized by vertical diffusion during incubation over night at 4 °C. A 200 µL standard stock solution containing 67 µg each of BSA, catalase, and thyroglobulin was applied to one tube and His_6/S-LmGMPR was overlayed in 200 µL volumes in the remaining tubes at concentrations of either 100, 500, or 1000 µg. Tubes were centrifuged at 38,000 rpm for 15 h in an Avanti ultracentrifuge with a SW-41 swinging bucket rotor. Fractions were collected from each tube in 0.4 mL volumes and precipitated with trichloroacetic acid. Samples of each fraction were analysed at 230 nm and 280 nm on a Beckman DU640 spectrophotometer as well as via SDS-PAGE.

2.7 In vivo MPA assay

The response of *L. donovani* promastigotes grown under strict purine conditions to the IMPDH inhibitor mycophenolic acid was assessed with a dose response assay. Promastigotes were seeded at a density of $1x10^5$ cells/200 µL in a 96 well microtiter plate and grown to stationary phase in DME-L or modified RPMI 1640 (Sigma) containing 100 µM hypoxanthine, xanthine, or guanine, and 5 % heat inactivated dialyzed filtered fetal bovine serum (dFBS). Negative controls were cells grown in media and 5 % dFBS but without supplemental purines. Two-fold serial dilutions of MPA over the range of 0.4-400 µM were set up for each purine to be used. Each set with a given purine was done in duplicate. The experiment was performed in triplicate. Cells grown in DME-L

were quantitated via visual enumeration using a hemacytometer and by absorbance at 655 nm (data not shown). Cells grown in modified RPMI 1640 media were quantitated via fluorescence of reduced Alamar Blue with a λ_{ex} of 545 and λ_{em} 590. Data was analyzed using Microcal Origins 7.0 software.

2.8 GTP/GMP binding and competition assays

Binding assays were performed with His_6/S-LmGMPR on both GMP and GTP immobilized agarose beads. HGPRT was used as the positive control and soybean trypsin inhibitor (TSI) (Boehringer Mannheim) as the negative control. Separate tubes containing 40 µL of packed beads were incubated with 20 µg of protein for 20 min at 20 °C. Each sample was washed 3 times with 0.5 mL of TBS. Samples of supernatant from each wash, as well as the beads, were retained for SDS-PAGE analysis.

Competition assays with GTP agarose beads were performed with untagged LmGMPR. Tubes containing 40 µL of packed GTP beads were incubated with 57 µg of LmGMPR for 5 min. ATP, GTP, or GMP were applied to the tubes in increasing concentrations (0, 0.1, 0.25, 0.5, 1, or 2.5 mM). Tubes were incubated with the effector for 20 min and then washed 3 times with 0.5 mL of TBS. Samples of supernatant and beads were retained for SDS-PAGE analysis.

2.9 Product detection via reverse phase HPLC

The forward and reverse LmGMPR enzymatic reactions were carried out at 20 °C for 1.5 hours in saturating concentrations of substrates. Standards, GMP, IMP, XMP, and NADPH, were prepared in GMP reductase assay buffer. A Beckman 32 KARAT High

Performance Liquid Chromatography machine was outfitted with a Supelco Discovery C18 column for the reverse phase analysis. The mobile phase consisted of a gradient of 2 buffers. Buffer A was 35 mM KH_2PO_4, 6 mM Tetrabutylammoniumhydrogensulphate (TBAS) pH 6 and buffer B contained a 1:1 v/v ratio of buffer A and methanol. The gradient went from 0-100% B over a 20 min period. Measurements were taken at 254 and 260 nm at a flow rate of 0.5 mL/min. Slight variability in retention times combined with close elution profiles precluded the differentiation of GMP from IMP based on retention time. As a result, purine nucleotides were distinguished via their characteristic absorption spectra. GMP exhibits two values for λ_{max}, 253 nm and 278 nm, while IMP has only one at 249 nm.

3. Results

3.1 Leishmania GMP reductase

Alignments of the *L. major* GMP reductase from the *L. major* gene database with other GMP reductases indicated a 5' sequence extension present on the putative gene. Based on alignment information with other GMP reductases, the 5' extension was omitted from the final PCR product by using primers targeting an internal site. Analysis of common domains revealed that the putative gene contained a GMP reductase/IMPDH signature sequence, cystathionine beta synthase (CBS) domains, a C-terminus peroxisomal targeting signal sequence-1 (PTS-1), and a mycophenolic acid (MPA) binding site (Fig. 6). CBS domains and MPA binding sites are characteristic of IMPDH enzymes. Thus far, no GMP reductase containing these domains has been described.

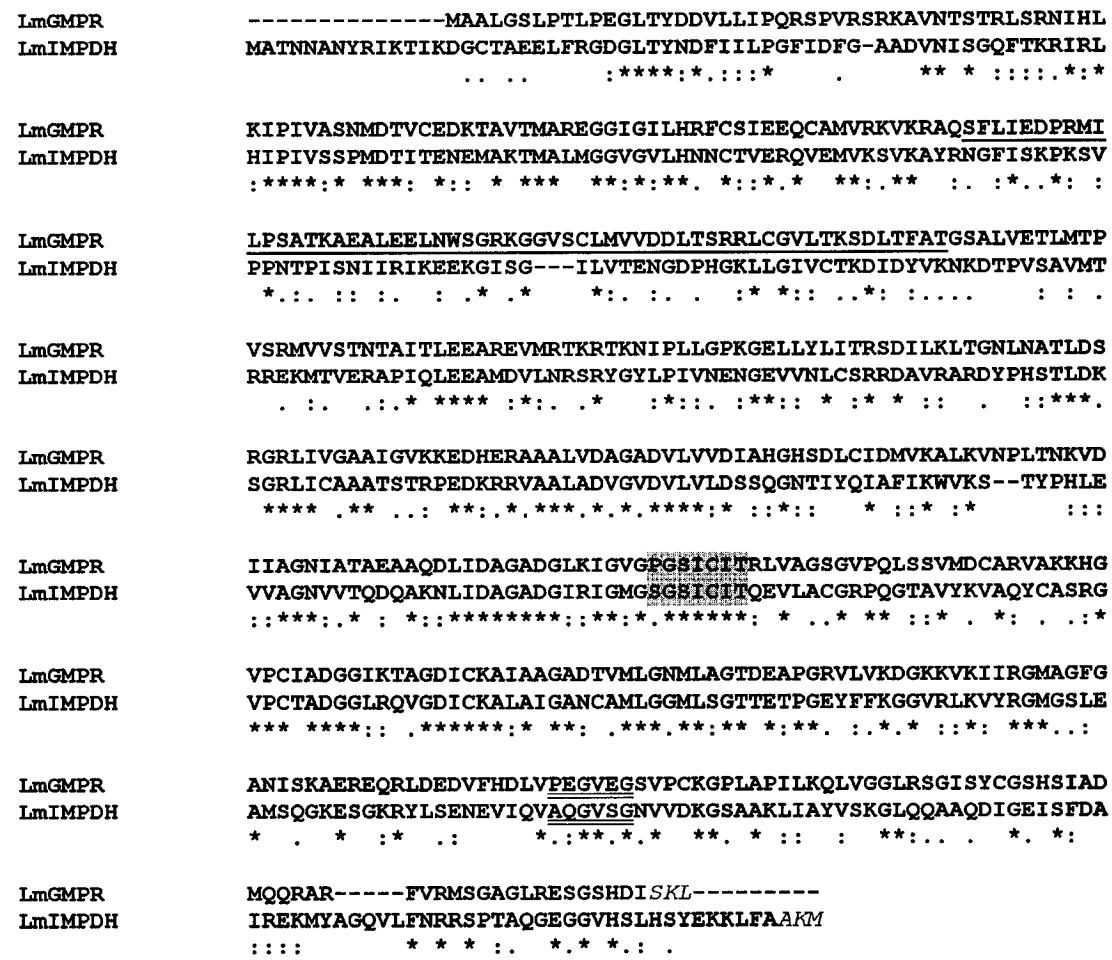

Figure 6. Clustal W sequence alignment of *L. major* IMPDH and GMPR. Underline: CBS domains, highlight: IMPDH/GMPR signature motif, double underline: MPA binding site, italics: Peroxisomal targeting signal sequence-1 (PTS-1).

The proteins with the highest identitities to LmGMPR, outside of the *Leishmania* and *Trypanosoma* species, are the putative IMPDHs of *Caldicellulosiruptor saccharolyticus* and *Syntrophomonas wolfei* subsp. wolfei str. Goettingen each with scores of 47% and positive identity values of 66% and 65%, respectively. Both of these organisms are obligate anaerobes belonging to the ancient Class of Clostridia. Interestingly, *S. wolfei* obtains its nutrients through β-oxidation of short chain saturated fatty acids (McInerney et al. 1981; Beaty et al. 1990). Sequence evidence that the *Leishmania* GMPR is glycosomally localized (presence of a PTS-1 sequence) along with

other purine metabolizing enzymes and the β-fatty acid oxidation pathway may suggest an evolutionary link between ancestors of these two organisms.

The GMP reductases of humans (GMPR2 isoform2) and *E. coli* K12 share 83% positive identity while scores between these two and LmGMPR are 58 and 59%, respectively (Fig. 7). Identity amongst IMPDHs is considerably less than that of GMP reductases when examined across organisms. Human IMPDH1 and *E. coli* K12 IMPDH share 59% positive amino acid identity. LmGMPR identities with each are 56 and 60%, respectively. Thus, the LmGMPR shares ~60% positive identity with both GMP reductases and IMPDHs. The prospect that this sequence similarity confers broader enzymatic capabilities is discussed further in this paper.

Overexpressed LmGMPR with a hexahistidine tag was purified from *E. coli ER2566* cells (Fig. 8). The recombinant enzyme migrates at an apparent molecular weight of 50 kD under the denaturing conditions of SDS-page but partitions in linear 5-20% sucrose gradients as a slightly larger entity than catalase, 250 kD (Fig. 9). This indicates that LmGMPR may be forming large complexes under native conditions. An untagged form of the enzyme has also been expressed and purified from a GMP reductase deficient cell line. Future work with this LmGMPR will assess what effect, if any, the N-terminal tag has on the oligomeric state of this enzyme.

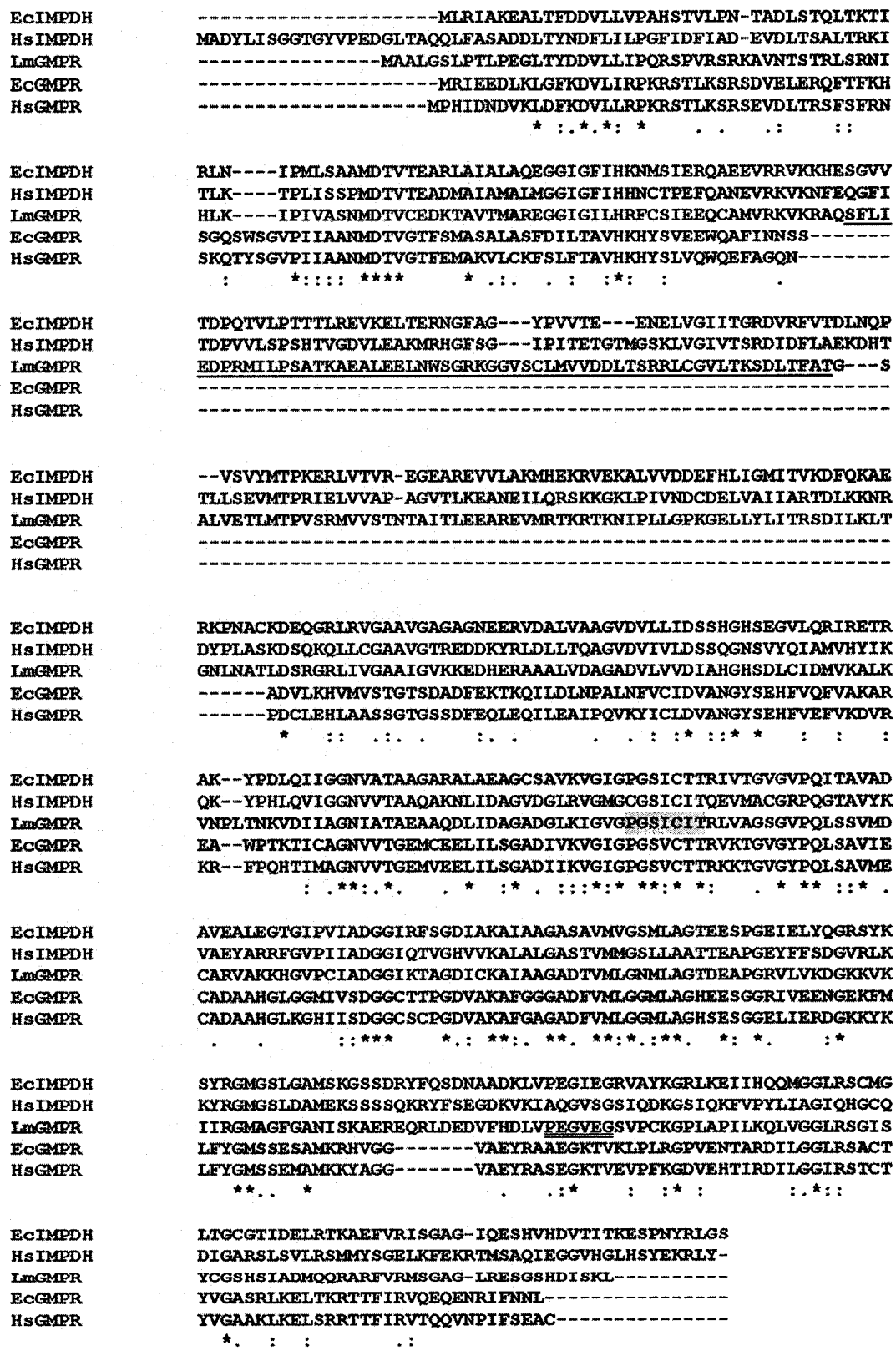

Figure 7. Clustal W sequence alignment of *E. coli* K12 IMPDH and GMPR, human IMPDH 1 and GMPR 2 isoform 2, with LmGMPR. Underline: CBS domains. Highlighted: IMPDH/GMPR signature motif. Double underline: MPA binding site.

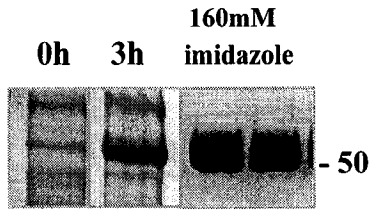

Figure 8. Induction and purification of LmGMPR. Enzyme was induced over 3 hours at 20°C with 0.6 mM IPTG and purified on a Ni^{2+} -NTA column with increasing concentrations of imidazole. The enzyme elutes in a pure fraction at 160 mM imidazole.

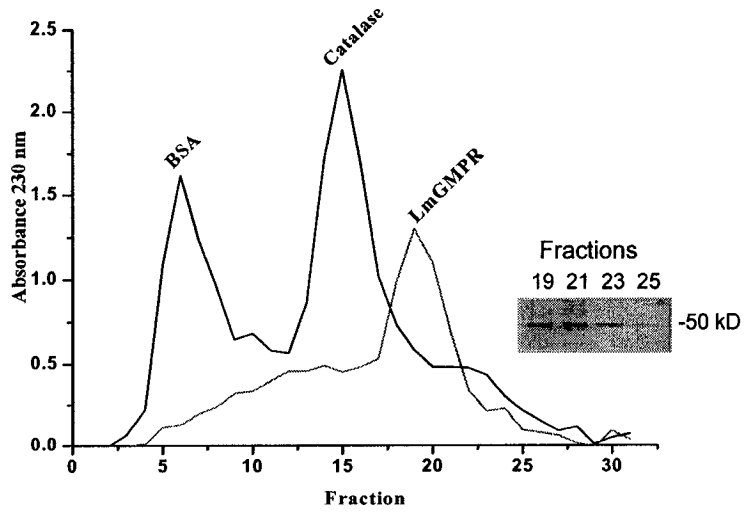

Figure 9. Quaternary structure of LmGMPR. Sucrose gradient fractionation of LmGMPR under non-reducing conditions reveals that the enzyme forms higher order aggregates. LmGMPR migrated as a slightly larger complex than catalase (250 kD).

3.2 Complementation

E. coli K12 cells lacking either the *IMPDH* (Δ *guaB*) or *GMPR* gene (Δ *guaC*) were transformed with the corresponding *Leishmania* gene and complementation of gene function was assessed qualitatively and quantitatively. For the Δ *guaC E. coli* auxotroph to grow in the presence of guanine the conversion of GMP to IMP is essential. This reaction is only catalyzed by GMP reductase. As demonstrated on plates supplemented with guanine as the sole purine source, the *E. coli* auxotrophs containing the *Leishmania GMPR* were capable of growing (Fig. 10-*2*). The plates were unable to support the

growth of cells containing only the plasmid (Fig. 10-*1*). From the liquid complementation studies it was determined that complemented GMPR activity was significant with a *p*-value of 0.001 (Fig. 11).

Due to considerable sequence similarity with the IMPDH the *Leishmania* GMPR was also assessed on its ability to catalyze the conversion of IMP to XMP. To investigate this it was necessary to use the Δ *guaB E. coli* cells (lacking IMPDH). IMPDH activity would restore the *E. coli* cells capacity to grow in the presence of hypoxanthine or adenine. Complementation of IMPDH activity by LmGMPR was not detected (Fig. 12). This may have been due to insufficient expression from the plasmid to drive the reaction.

It was possible to complement IMPDH deficiency in Δ *guaB* cells with the *L. donovani IMPDH* gene as demonstrated by the bacterial growth in the presence of hypoxanthine (Fig. 10-*4*). The plasmid alone in Δ *guaB* cells could not support growth on this media (Fig. 10-*3*). Growth of pBAce-*LdIMPDH* Δ *guaB* cells was significant with a *p*-value of 0.003 (Fig. 13). In addition, GMPR activity could not be detected in GMP reductase deficient cells transformed with the LdIMPDH.

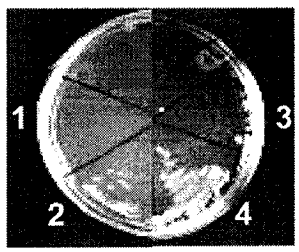

Figure 10. Complementation of LmGMPR and IMPDH in auxotrophic *E. coli* K12 cells. *E. coli* Δ *guaC* and *guaB* cells lack GMP reductase and IMPDH, respectively. Modified arabinose induction media containing 35 μg/mL guanine: 1- pBAD Δ *guaC*; 2- LmGMPR pBAD Δ *guaC*. Low phosphate induction media containing 50 μg/mL hypoxanthine: 3- pBAce Δ *guaB*; 4- LdIMPDH pBAce Δ *guaB*. LmGMPR complements GMP reductase activity. The LdIMPDH also restores the IMPDH phenotype in *E. coli* auxotrophs.

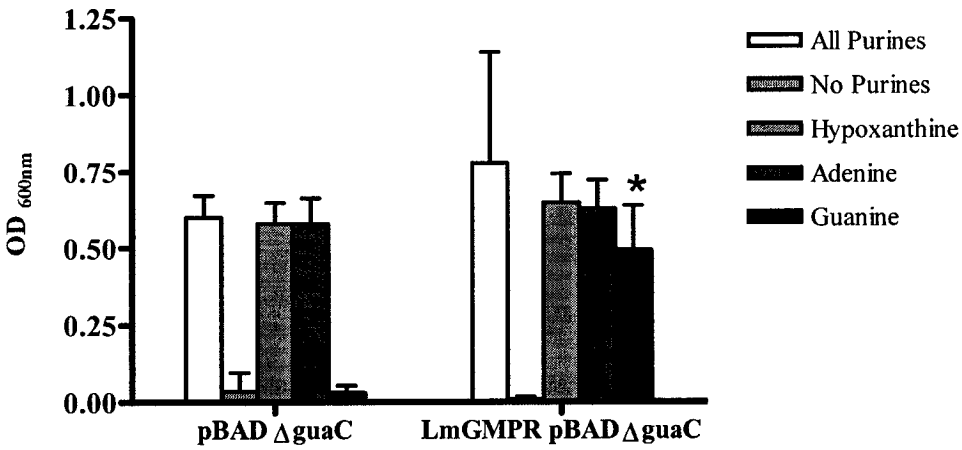

Figure 11. Quantitative analysis of GMPR complementation by LmGMPR. GMP reductase deficient *E. coli* cells (Δ *guaC*) containing plasmid expressing LmGMPR are capable of growing in the presence of guanine. Cells transformed with the vector alone do not grow. Significance (*) was determined via the Student's T-test. p= 0.001

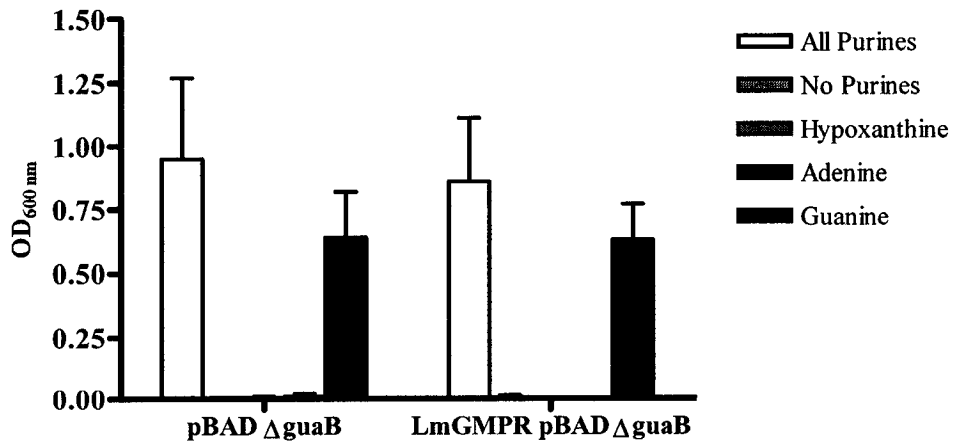

Figure 12. Quantitative analysis of IMPDH complementation by LmGMPR. The *Leishmania* GMPR is incapable of complementing IMPDH function in IMPDH-deficient *E. coli* cells (Δ *guaB*) as demonstrated by the inability to grow in the presence of hypoxanthine or adenine.

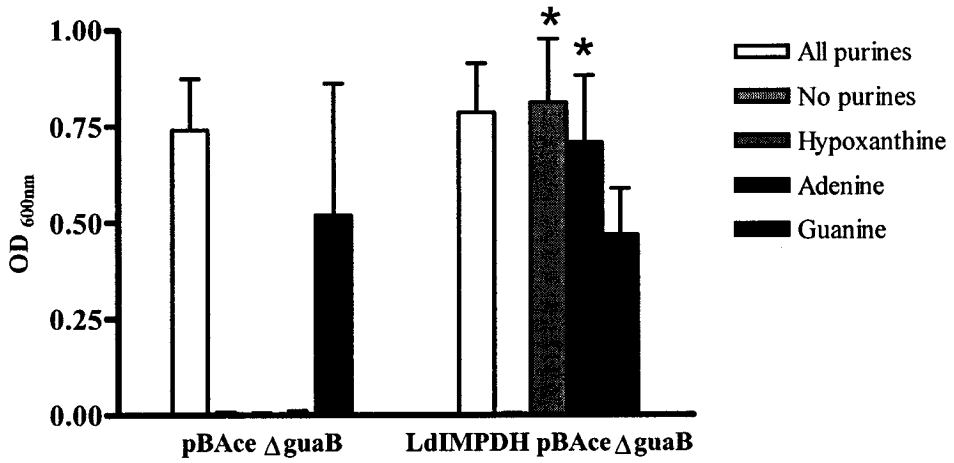

Figure 13. Quantitative analysis of IMPDH complementation by LdIMPDH. IMPDH deficient *E. coli* cells (Δ *guaB*)containing plasmid expressing LdIMPDH are capable of growing in the presence of hypoxanthine and adenine. Cells transformed with the vector alone do not grow. Significance (*) was determined via the Student's T-test. p= 0.003

3.3 Kinetics

Both tagged and untagged forms of LmGMPR slightly deviated from Michaelis-Menten behaviour with regard to GMP but not with NADPH. Sigmoidicity of the Michaelis-Menten plot was not initially apparent (Fig. 14). The non-hyperbolic behaviour was eventually observed from Scatchard-Eadie and Hanes-Woolf transformations (Fig. 15). Interestingly, this sigmoidicity is abrogated in the presence of 200 μM NADPH, which is 20 times the calculated K_m for NADPH (Fig. 16). In contrast, LmGMPR appears to behave according to Michaelis-Menten kinetics with regard to NADPH (Fig. 17).

In the case of GMP, calculations of K_m and V_{max} have been done via the direct linear method and corroborated with Hanes-Woolf transformations plotted with the initial rate data for GMP concentrations equal to and greater than the K_{mGMP}. Since LmGMPR obeys Michaelis-Menten kinetics with regard to NADPH, rate data from all NADPH

concentrations were used in the determination of K_m and V_{max}. His_6/S-LmGMPR was found to oxidize NADPH in the presence of GMP with calculated K_m values of 20 μM for GMP and 6 μM for NADPH (Fig. 18 & 19). The forward GMPR reaction does not appear to utilize NADH as an alternative electron donor given that no rates were detected up to 100 μM NADH. The turnover rate for the forward reaction of His_6/S-LmGMPR was 0.009/min for both NADPH and GMP. His_6/S-LmGMPR appears to catalyze an essentially irreversible reaction as there was no detectable rate for the reverse GMP reductase reaction. Product formation for the forward reaction was confirmed via reverse phase HPLC, however this method did not produce evidence of the reverse GMPR reaction (data not shown). His_6/S-LmGMPR did not appear to be affected by excess IMP, GMP, or XMP. GTP and ATP were investigated as potential effector molecules for the tagged LmGMPR, however, this form of the enzyme was not affected by GTP at all and showed minimal inhibition by ATP.

In contrast, kinetics performed with the untagged form of the enzyme indicate the importance of the N-terminal end for enzyme function. Non-hyperbolic behaviour was still evident for the untagged form of LmGMPR with regard to GMP (Fig. 20). Estimated calculations of K_m and V_{max} for GMP have been done via Hanes-Woolf plots containing the initial rate data from the $\geq K_m$ GMP concentrations as well as by curve fitting directly to the data. Michaelis-Menten behaviour by LmGMPR with regard to NADPH permitted the use of standard linear transformations of the equation for K_m and V_{max} determination. The K_m values for both GMP and NADPH, derived from the Hanes-Woolf plots, remained similar whereas the calculated turnover rate increased by ~6-fold.

The K_ms for GMP and NADPH were 37 and 12 μM, respectively (Fig. 21 & 22). The turnover rate was calculated to be 0.059/min for GMP and 0.043/min for NADPH.

Assessment of GTP activation and inhibition by IMP and ATP on LmGMPR has been based on the initial rate plots. The sigmoidicity of the data precludes the determination of kinetic constants based on linear transformations of the Michaelis-Menten equation. For further discussion of the sigmoidal plots the term $S_{0.5}$ will be used to refer to the substrate concentration at ½ V_{max}. By definition K_m is the Michaelis-Menten constant and thus cannot technically be utilized when referring to alternate kinetic behaviour. The plateau of the sigmoidal plots is referred to as apparent V_{max}.

Contrary to the His_6/S-LmGMPR, the untagged form of the enzyme displayed product inhibition by IMP (Fig. 23). The apparent V_{max} decreased in the presence of 1 mM IMP by 72 %. GTP also was observed to be a potent activator of untagged LmGMPR at GMP concentrations near the $S_{0.5}$. Reaction velocity increased by up to 400% at 20 μM GMP in the presence of 10 μM GTP (Fig. 24). In contrast, ATP exerted an inhibitory effect on LmGMPR. The effect with GMP was much more potent than with NADPH. The $S_{0.5}$ of GMP was doubled and the V_{max} was reduced by 25 % in the presence of 25 μM ATP (Fig. 25). Moreover, the sigmoidicity of the initial rate data was enhanced in the presence of ATP. It required a much higher concentration of ATP to exert a noticeable effect on LmGMPR under varied NADPH and saturating GMP concentrations. At ATP concentrations of 100 μM and higher the $S_{0.5}$ for NADPH increases and the V_{max} decreases (Fig. 26). At 250 μM the $S_{0.5}$ is two-fold higher and the V_{max} is reduced by 34 %.

Due to substantial sequence similarity it was crucial to investigate whether the LmGMPR was capable of catalyzing the IMPDH reaction. With the recombinant enzyme it was observed that after a ~60 s lag period there was detectable reduction of NAD^+ in the presence of IMP and 500 nM LmGMPR (Fig. 27). This suggests that it may be possible to drive the LmGMPR to perform the IMPDH reaction. There are two caveats to this observation: the 4.9 kD hexahistidine tag may have sufficiently altered the flexibility of the enzyme hindering its ability to efficiently perform the reaction and resulting in the confounding lag period; and contaminating *E. coli ER2566* IMPDH from the expression process may have influenced the rates. The possibility of *E. coli* IMPDH persisting on the Ni^{2+} -NTA column throughout the stringent washing process of the purification is low however one cannot rule out the possibility of hetero-oligomer formation between LmGMPR and endogenous *E. coli* IMPDH monomers. Interestingly, the IMPDH reverse reaction has been undetectable.

Specific amino acid residues are critical components of the catalytic mechanism. To elucidate which amino acids participate in the reaction catalyzed by LmGMPR, modifying agents were applied to the enzyme and activity was assessed. Chemicals such as n-ethylmaleimide (NEM), diethyl pyrocarbonate (DEPC), and diacetyl, which selectively modify specific amino acid residues, were incubated with the enzyme to elucidate the critical residues for enzyme function. NEM covalently reacts with available sulfhydryl groups. This chemical rapidly and completely inactivated LmGMPR. A critical cysteine at position 319 within the IMPDH/GMPR domain is the suspected residue contributing to complete loss of activity in the presence of NEM. Arginine residues do not appear to play a direct role in catalysis since diacetyl did not exert an

effect on the enzyme activity. DEPC however did alter enzymatic activity suggesting that a histidine residue is also important in the LmGMPR reaction (Fig. 28).

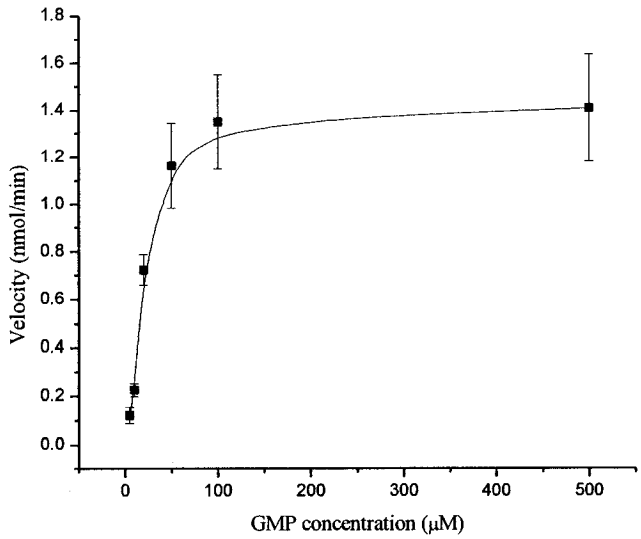

Figure 14. His_6/S-LmGMPR activity in the presence of varied GMP (20-500 μM) and 45 μM NADPH. Kinetic data is fit to the Michaelis-menten equation. Very slight sigmoidicity was undetected on this plot. As a result linear transformations of the data were used to obtain kinetic parameters for GMP.

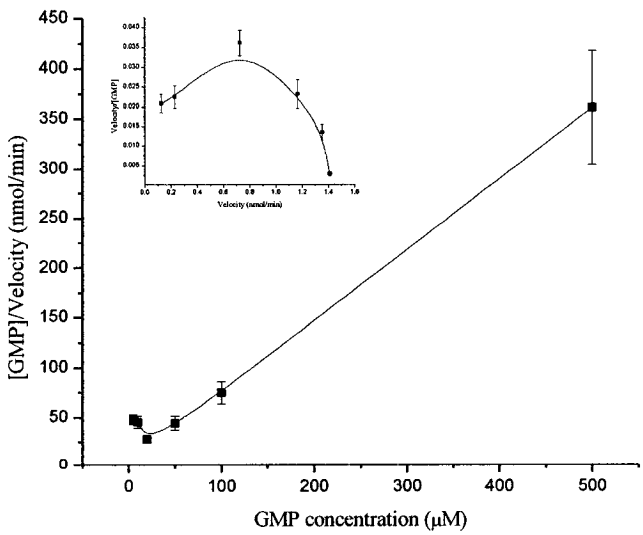

Figure 15. Non-hyperbolic nature of LmGMPR with regard to GMP. His_6/S-LmGMPR was assayed under varying GMP conditions (20-500 μM) and fixed NADPH (45 μM). Data was plotted via the Hanes-Woolf transformation and the Scatchard-Eadie plot (inset). These results are characteristic of either co-operativity or a deviant bi-substrate reaction mechanism.

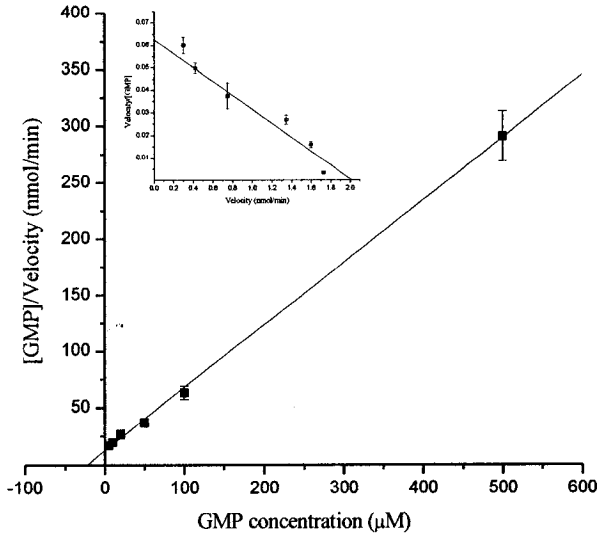

Figure 16. His_6/S-LmGMPR activity in the presence of varied GMP (20-500 μM) and fixed NADPH (200 μM) Data is presented via the Hanes-Woolf method and the Scatchard-Eadie plot (inset). Concentrations of NADPH of 20X K_m (200 μM) abrogate the non-hyperbolic kinetics observed at lower NADPH concentrations.

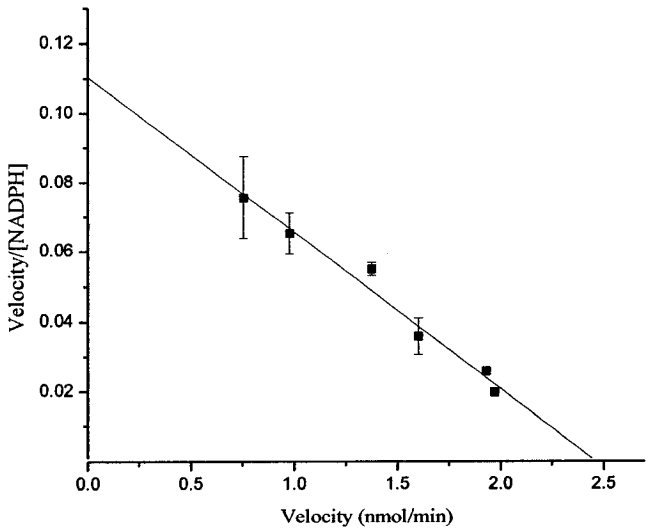

Figure 17. Untagged LmGMPR displays Michaelis-Menten behaviour with regard to NADPH. Assays were performed with 300 μM GMP and varied NADPH (10-100 μM). The Scatchard-Eadie plot shows no deviation from linear rates.

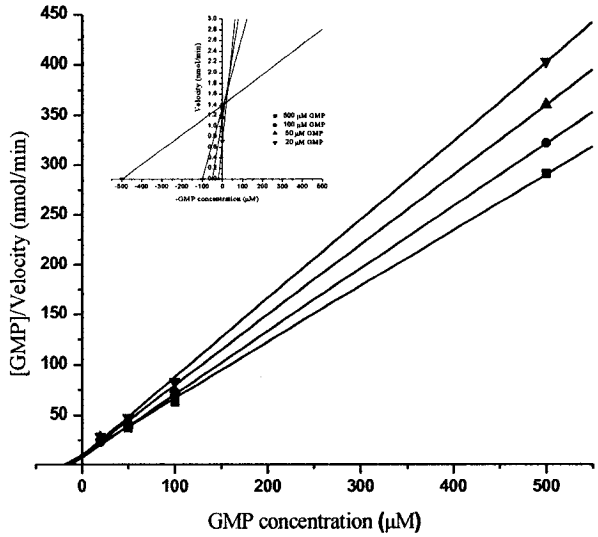

Figure 18. Kinetic characterization of His_6/S-LmGMPR with respect to GMP. ■ 200 μM NADPH • 100 μM NADPH ▲ 50 μM NADPH ▼ 20 μM NADPH. Direct linear (inset) and Hanes-woolf plots revealed the K_m for GMP to be 20 μM. The K_{cat} is 0.009/min.

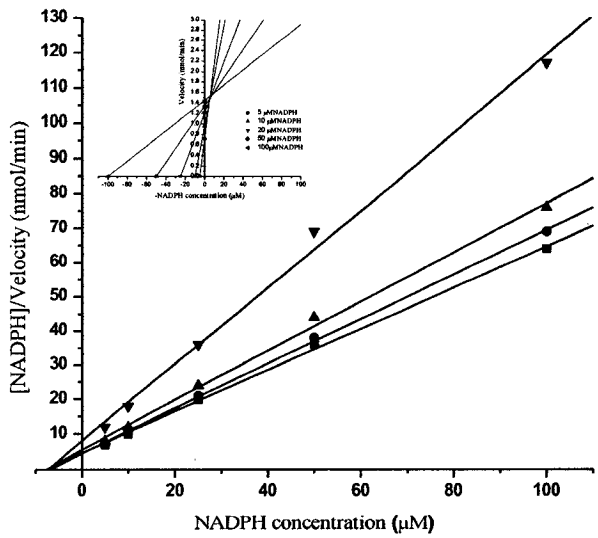

Figure 19. Kinetic characterization of His_6/S-LmGMPR with respect to NADPH. ■ 500 μM GMP • 100 μM GMP ▲ 50 μM GMP ▼ 20 μM GMP. Direct linear (inset) and Hanes-woolf plots revealed the K_m for NADPH to be 6 μM. The K_{cat} is 0.009/min.

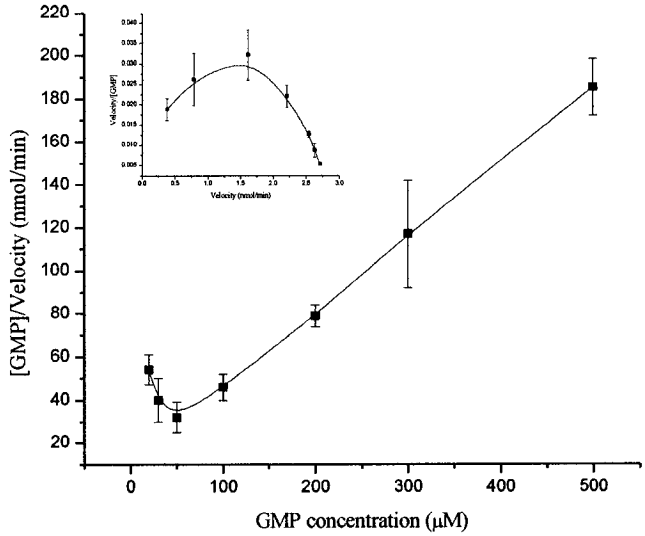

Figure 20. Non-hyperbolic behaviour of the untagged LmGMPR. Assays performed under varied GMP (20-500 μM) and constant NADPH (100 μM) with 46 nM LmGMPR indicate co-operativity or deviant bi-substrate kinetics.

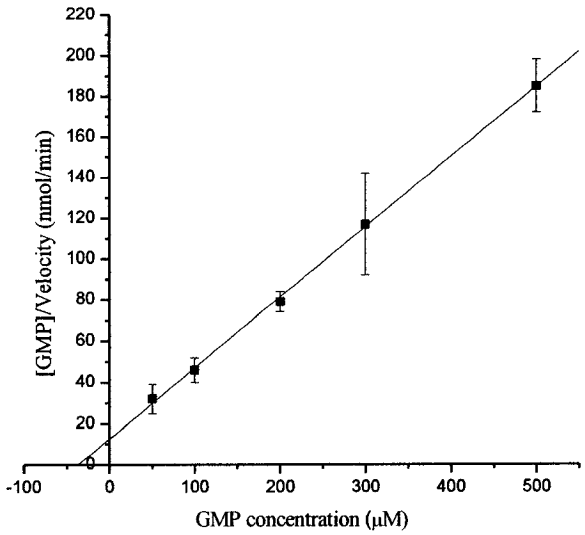

Figure 21. Hanes-Woolf of untagged LmGMPR under saturating substrate conditions. GMP concentration was varied (20-500 μM) while NADPH was kept constant at 100 μM. LmGMPR was used at a concentration of 46 nM. Determined K_m for GMP is 37 μM and the K_{cat} is 0.059/min.

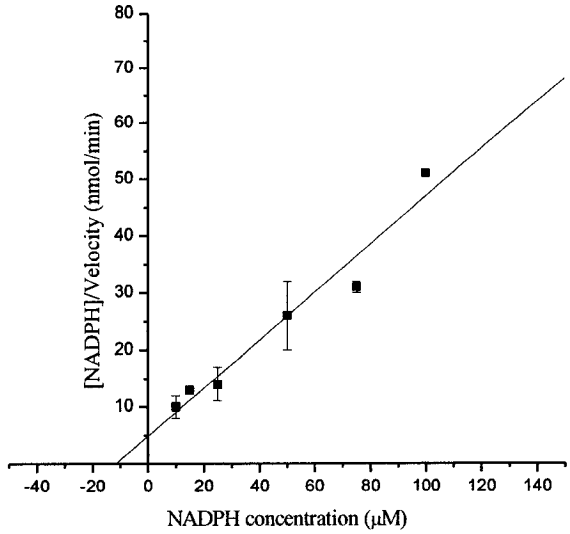

Figure 22. Hanes-woolf of untagged LmGMPR under saturating substrate conditions. NADPH concentration was varied while GMP was kept constant at 300 μM. LmGMPR was used at a concentration of 46 nM. The determined K_m for NADPH is 12 μM and the K_{cat} is 0.043/min.

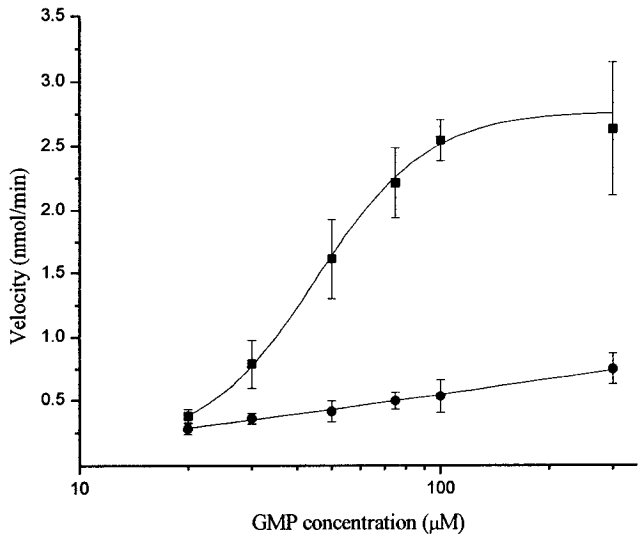

Figure 23. Untagged LmGMPR inhibition with IMP. Kinetic assays were conducted with varied GMP concentration (20-300 μM), 100 μM NADPH, and 46 nM LmGMPR. ■ 0 μM IMP • 1 mM IMP. LmGMPR exhibits product inhibition by IMP.

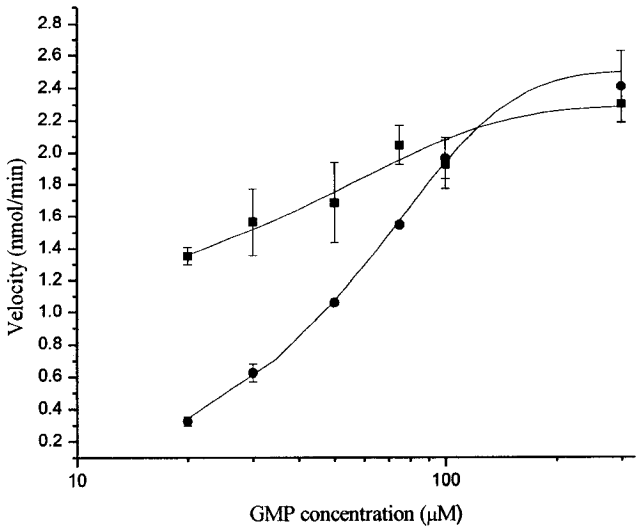

Figure 24. GTP activation of untagged LmGMPR. Kinetic assays were performed with 100 μM NADPH, 46 nM LmGMPR, varied concentrations of GMP (20-300 μM), in 75 mM sodium phosphate buffer pH 6.9 with 1 mM EDTA, 1 mM DTT, and 100 mM KCl. ■ 10 μM GTP • 0 μM GTP. In the presence of GTP the reaction velocities near the K_m for GMP and below are elevated. Under saturating conditions of GMP LmGMPR retains the same V_{max}.

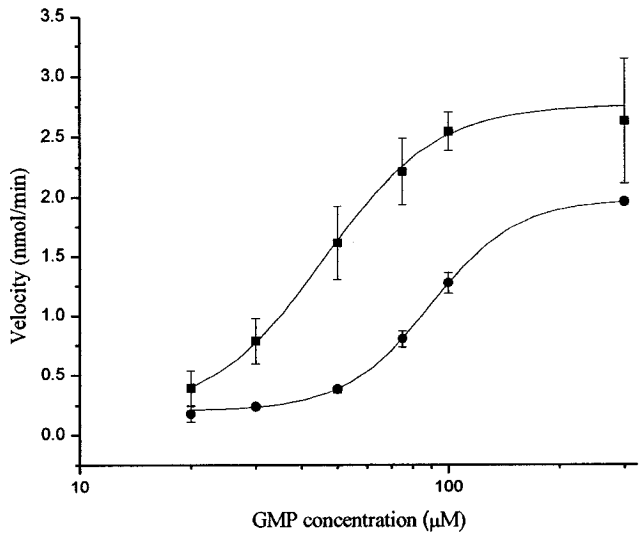

Figure 25. ATP inhibition of untagged LmGMPR. Kinetic assays were performed with 100 μM NADPH, 46 nM LmGMPR, varied concentrations of GMP (20-300 μM), in 75 mM sodium phosphate buffer pH 6.9 with 1 mM EDTA, 1 mM DTT, and 100 mM KCl. ■ 0 μM ATP • 25 μM ATP. In the presence of ATP the $S_{0.5}$ for GMP increases to ~90 μM and the V_{max} decreases by 28%.

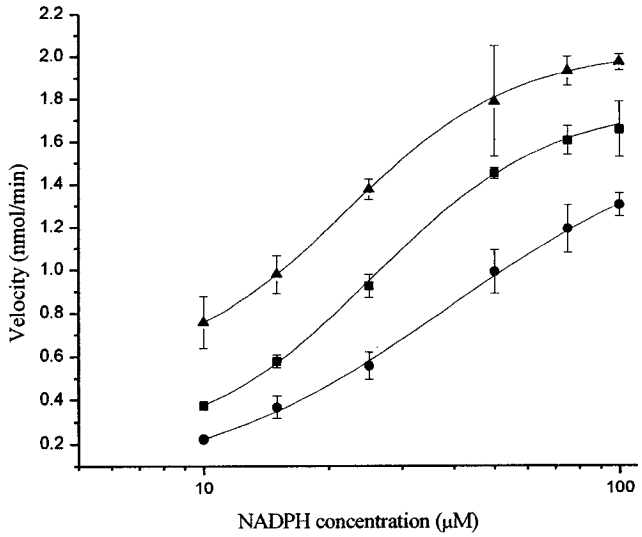

Figure 26. ATP inhibition of untagged LmGMPR in relation to NADPH. Kinetic assays were performed with 300 μM GMP, 46 nM LmGMPR, varied concentrations of NADPH (10-100 μM), in 75 mM sodium phosphate buffer pH 6.9 with 1mM EDTA, 1mM DTT, and 100 mM KCl. ▲ 0 μM ATP ■ 100 μM ATP • 250 μM ATP. In the presence of 250 μM ATP, reaction velocities are decreased by 56-75%.

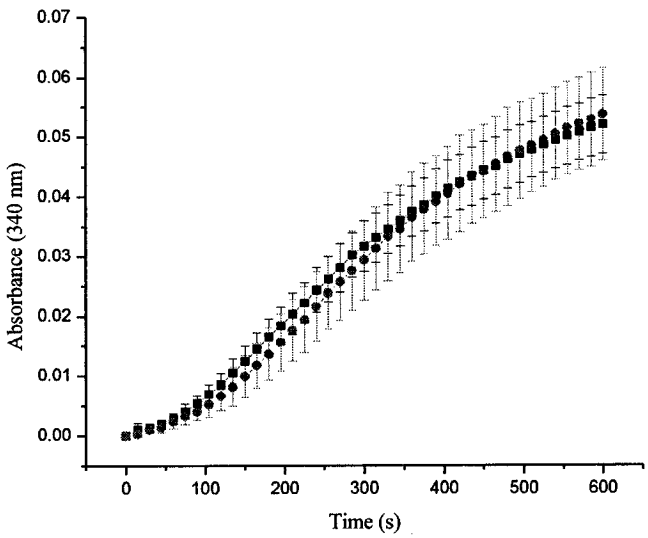

Figure 27. IMPDH activity of LmGMPR. ■ 10 μM IMP • 100 μM IMP. Reduction of NAD^+ occurs in the presence of 10-100 μM IMP, 100 μM NAD^+ and 927 nM His_6/S-LmGMPR. Velocity slows as the NAD^+ concentration is lowered.

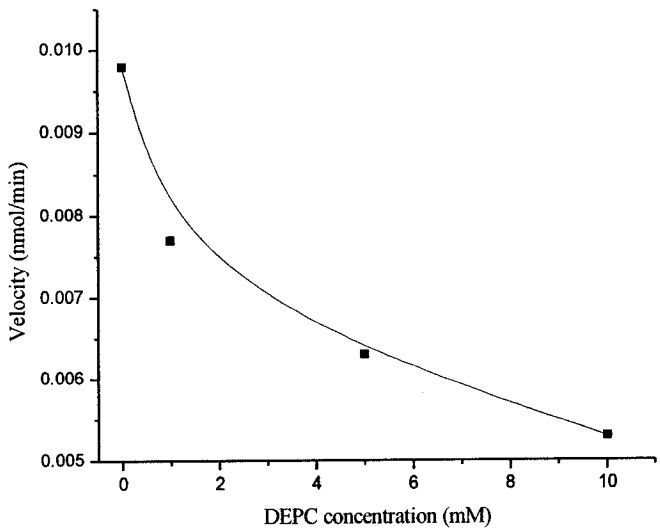

Figure 28. DEPC inactivation of untagged LmGMPR. Enzyme was incubated with specified DEPC concentrations for 15 min and assayed with 300 μM GMP, 100 μM NADPH and 46 nM LmGMPR. DEPC affects the activity of LmGMPR. The enzyme probably utilizes a histidine residue in its catalytic mechanism.

3.4 GMP/GTP binding characteristics

Binding assays performed with His_6/S-LmGMPR and the untagged enzyme demonstrate that both forms bind GMP and GTP agarose beads. The binding is stringent enough to persist through multiple washes (Fig. 29). Since GTP does not act as an inhibitor for GMP and it cannot be utilized as a substrate it likely binds to a different location on the enzyme. Based on evidence that the CBS domains are important regulatory domains that bind ATP (Scott et al. 2004) it was hypothesized that its binding site on LmGMPR may be within the CBS domain . Structural similarities between ATP and GTP may permit binding of GTP to the CBS domain also. Competition assays have been conducted with GTP agarose to elucidate the binding site for each on the untagged LmGMPR. Excess ATP, GMP and GTP were used up to 2.5 mM to compete for the GMPR regulatory binding site. GMPR bound the GTP beads tightly however no reduction in binding was seen even at the highest concentration of effector used (Fig. 30).

ATP and GTP may bind mutually exclusive sites on the enzyme. However, the LmGMPR remained bound to GTP beads even in the presence of excess free GTP. These binding experiments do not discount the possibility that LmGMPR may form a covalent intermediate with GMP or GTP. In this circumstance, LmGMPR may not dislodge from the beads in the presence of excess ligand. Alternatively, the binding of GTP by LmGMPR may be very strong. Addition of free ligand after LmGMPR has bound to the beads may not be sufficient to remove the enzyme. Reversing the order of the experiment, by incubating LmGMPR with free effector then applying it to the beads, may yield more conclusive results.

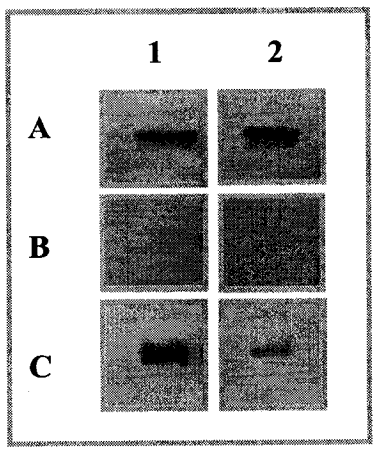

Figure 29. LmGMPR binding to GTP and GMP agarose beads. A1: hypoxanthine-guanine phosphoribosyltransferase (HGPRT), GTP beads. A2: HGPRT, GMP beads. B1: soybean trypsin inhibitor (TSI), GTP beads. B2: TSI, GMP beads. C1: His_6/S-LmGMPR, GTP beads. C2: His_6/S-LmGMPR, GMP beads.

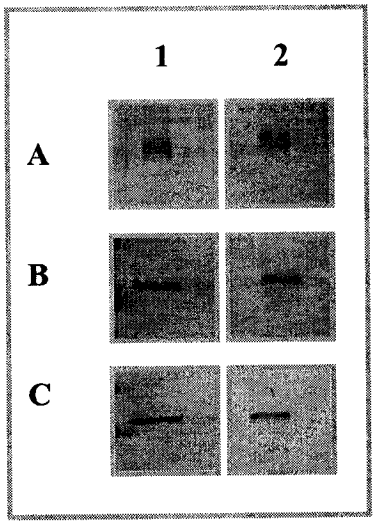

Figure 30. Competition assay with LmGMPR and GTP beads. A1, B1, C1: GTP beads loaded with LmGMPR, A2: GTP beads after competition with excess GMP and three washes, B2: GTP beads after competition with excess GTP and three washes, C2: GTP beads after competition with excess ATP and three washes.

3.5 MPA inhibition

LmGMPR is unique in that it contains a putative MPA binding site. This site has higher identity to MPA binding sites of prokaryotic IMPDHs than eukaryotic IMPDHs. No GMP reductases have been described to date that contain identifiable MPA binding sites. To investigate whether the LmGMPR MPA binding site was functional, kinetic assays were performed with His_6/S-LmGMPR. MPA inhibits IMPDH enzymes by competing with NAD^+ for its binding site and halting the release of the product, XMP, by physically interfering with the enzymatic mechanism (Sintchak et al. 1996). Hanes-woolf and Lineweaver Burk plots show an increase in K_m and a stationary V_{max} indicating that MPA is a competitive inhibitor of LmGMPR with respect to NADPH (Fig. 31). To determine the K_i of MPA the transformation of K_{mapp}/V_{max} versus MPA concentration was used (Kakkar et al. 1999). This plot gave a K_{iMPA} of 20 μM which corresponds to

those of microbial IMPDH enzymes containing the same weak MPA binding site sequence, **PEG(I/V)EG**.

In vivo dose response assays were conducted under strict purine conditions to determine the EC_{50} of this drug on the IMPDH and the GMPR (Fig. 32). Supplying *L. donovani* promastigotes with only hypoxanthine permitted isolation of the effect of MPA on the IMPDH. The EC_{50} of MPA on the IMPDH is 1.7 ± 0.14 μM. This 100-fold increase in concentration from the MPA K_i (which is ~10-20 nM) for IMPDH is likely a result of dilution and uptake effects. By restricting the supply of purines to xanthine or guanine it was possible to isolate the effect of MPA on the GMP reductase. Concentrations as high as 400 μM MPA do not show an effect on the growth of *L. donovani* promastigotes grown in guanine or xanthine. This is not unexpected since the

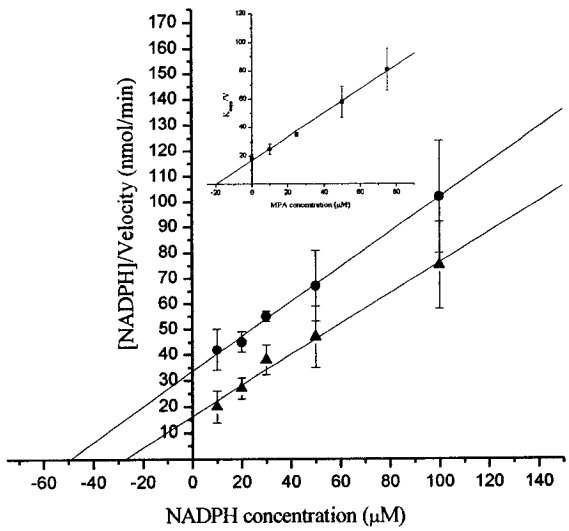

Figure 31. Effect of mycophenolic acid (MPA) on LmGMPR. ▲ 0 μM MPA • 25 μM MPA. Data plotted via the Hanes-Woolf linear transformation indicates that MPA is a competitive inhibitor of LmGMPR with respect to NADPH. Plotting the intercepts yields a K_i of 20 μM (inset).

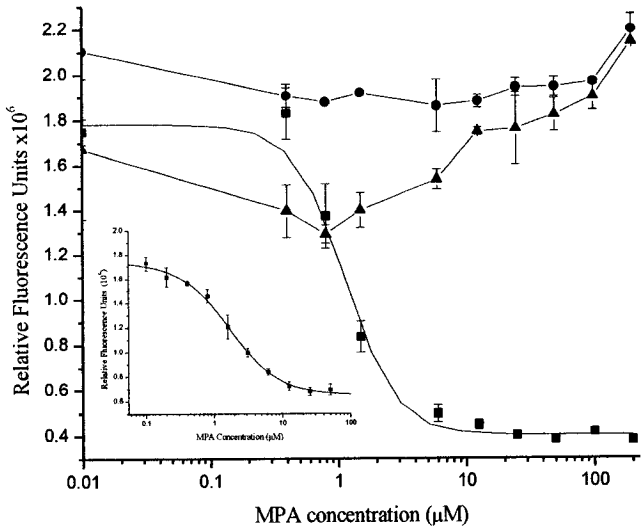

Figure 32. *In vivo* MPA dose response assay. ■ 100 μM hypoxanthine • 100 μM guanine ▲ 100 μM xanthine. *Leishmania donovani* promastigotes cultured in the presence of increasing concentrations of MPA and hypoxanthine display growth inhibition. The EC_{50} of MPA on the IMPDH is 1.7 ± 0.14 μM (inset). Culturing cells in the presence of MPA and only xanthine or guanine does not result in growth inhibition. Thus, MPA does not affect the GMPR at these concentrations.

in vivo distortion of K_i would be of the same magnitude on the GMPR as with the IMPDH. Since the LmGMPR K_{iMPA} is 20 μM, the EC_{50} for parasites grown solely in guanine might be expected to be as high as 4 mM MPA.

4.0 Discussion

Leishmania species rely exclusively on salvage mechanisms to acquire purines from their host. Efficient incorporation of these molecules into the purine metabolic pathway requires a multitude of specialized enzymes. GMP reductase fulfills the critical role of maintaining balance between the GMP and AMP pools. For organisms with *de novo* synthesis capabilities this serves as a back-up system, however for auxotrophic

organisms such as *Leishmania* this enzyme is crucial for adaptation to and survival within nutritionally diverse environments.

Prior work by Spector and Jones (1982) indicated the presence of GMP reductase activity in crude lysates of *Leishmania donovani* promastigotes. However, the gene encoding *Leishmania* GMP reductase remained unidentified. This work has set out to identify the *Leishmania* GMP reductase gene, express and purify the protein, and biochemically characterize its enzymatic activity.

Belonging to the family of enzymes involved in guanine nucleotide biosynthesis, IMPDHs and GMP reductases share a significant amount of sequence homology. The presence of an uncharacterized, "putative" IMPDH gene in the *L. major* genome gave credence to the hypothesis that *Leishmania* species did indeed utilize a GMP reductase in their purine metabolic pathway. Examination of the amino acid architecture of this protein revealed interesting deviations from other known GMP reductases. This protein contained CBS domains and an MPA binding site, which are IMPDH-specific characteristics, and a C-terminal PTS-1 signal sequence, suggesting glycosomal localization. Cloning of the *GMPR* gene from the *L. major* genome into various vectors has enabled the expression and purification of the His_6/S tagged and untagged enzyme. In this research, both the recombinant and the endogenous form of the enzyme have been utilized in the investigation of function and regulation of the LmGMPR.

The recombinant enzyme contained an N-terminal 5 kD hexahistidine/S tag. Based on prior published data and sequence analysis indicating that the catalytic pocket was located at the C-terminal end it was hypothesized that the positioning of this tag at the N-terminus might avoid any major alterations to enzyme behaviour (Farber et al.

1990; Bork et al. 1995). This is not the case. The *Leishmania* GMP reductase is complex in its catalytic function and regulation and the presence of the affinity tag had serious implications on its activity. The turnover rate increased by 6-fold from 0.009/min for His_6/S-LmGMPR to 0.059/min for untagged LmGMPR. In addition, response to effector molecules, such as ATP and GTP was abrogated by the N-terminal tag. However, the presence of the tag appeared not to affect the K_m values for NADPH and GMP. Maintaining flexibility in the N-terminus appears to be crucial for proper functioning of the LmGMPR. Since K_{cat} was drastically reduced and the K_m values were unaffected it suggests that tagged LmGMPR was able to bind both substrates but was incapable of efficiently performing the catalysis step.

The kinetic activity of the LmGMPR appears to be highly complex. The enzyme exhibits an unusual kinetic pattern at NADPH concentrations at or below 10-fold K_m under conditions of sub-saturating GMP. Velocity appears to be disproportionately low at GMP concentrations below the K_m and NADPH concentrations of 100 μM or less. This can be described by either positive co-operativity (Neet 1996) or as a complexity of bi-substrate kinetic activity (Ferdinand 1966). A number of graphical methods have been employed to illustrate this deviation from Michaelis-Menten kinetics. It is indicated by the parabolic curve on a Scatchard-Eadie plot (V/S against V) and the characteristic "check mark" on the Hanes-woolf plot.

Enzymes obeying Michaelis-Menten kinetics require an 81-fold increase in substrate concentration to traverse from 10-90% of V_{max}. Typically, positive co-operativity is described by a substantial decrease in the amount of substrate necessary to attain this corresponding rate increase. Analysis of the Michaelis-Menten data of

reactions in the presence of varied GMP indicates that at NADPH concentrations less than or equal to 100 μM LmGMPR requires a ~14-fold increase in GMP to reach 90% of V_{max}. In this circumstance, GMP may act as a homotropic modulator altering the conformation of adjoining subunits to accommodate GMP binding more readily.

Although, non-hyperbolic rate curves can be indicative of co-operativity they may also represent complex enzymatic behaviour resulting from the bi-substrate mechanism of LmGMPR (Ferdinand 1966). This alternate hypothesis describes a situation in which both substrates are capable of binding to the free enzyme, forming a ternary complex, and proceeding to product formation. However, one substrate is capable of binding free enzyme more efficiently than the other. In the case of LmGMPR, at constant NADPH and low GMP levels the formation of the E-NADPH complex may be less efficient but will predominate due to the higher prevalence of this substrate in the reaction mixture. As a result, most of the GMP will bind the E-NADPH complex as opposed to free enzyme and the reaction velocity at these substrate concentrations will be slower. When the GMP concentration is elevated it binds to LmGMPR in a kinetically favourable manner resulting in the predominant formation of the E-GMP complex. This corresponds to the increase in velocity observed at higher GMP concentrations.

This deviation from Michaelis-Menten behaviour is extremely beneficial for enzymes that need to respond to fluctuating levels of substrate in the environment (Gibson 1970; Bontemps et al. 1978). The digenetic lifecycle of *Leishmania* parasites exposes them to a diverse array of nutrients thus requiring a certain degree of flexibility in their metabolism. The LmGMPRs enzymatic mechanism appears to be efficient at responding to variable purine levels. Under low GMP concentrations the LmGMPR does

not efficiently shunt GMP back to IMP. When the pool of GMP increases the enzyme begins catalyzing the reaction. This form of regulation would be crucial for maintaining a balanced pool of purines within the cell.

Surprisingly, at very high NADPH concentrations the lag in rate is abolished and the velocity becomes linear with respect to GMP concentration. This may be an artifact of non-physiological conditions. To date, it is unknown what the concentration of NADPH is within the glycosome. Conversely, NADPH concentration in the glycosome may never go below 200 μM making the previous observation of non-hyperbolic behaviour inconsequential. Interestingly, at constant GMP (5-10-fold K_m) and varying NADPH LmGMPR exhibits characteristic Michaelis-Menten kinetics. This observation does not negate the previous hypotheses regarding LmGMPRs non-hyperbolic nature with respect to GMP. Both explanations allow for the enzyme to conform to Michaelis-Menten behaviour at high GMP and varied NADPH concentrations. In the case of co-operativity, it is possible that LmGMPR only responds in this manner to the binding of GMP. For the alternate bi-substrate mechanism, the predominance of fixed saturating levels of GMP would lead to the preferential formation of the E-GMP complex prior to NADPH binding. The GMP-dependent lag phase observed before would be abolished under these conditions. The kinetic activity of the LmGMPR is very complex and appears to be highly sensitive to substrate shifts in the environment. This suggests that the LmGMPR is a crucial site for regulation of the purine metabolic pathway in *Leishmania.* Further kinetic studies will reveal the mechanism of the non-hyperbolic activity, the effect of "super" saturating concentrations of NADPH on the LmGMPR, and the physiological relevance of this sigmoidal phenotype.

Beyond the complex primary kinetic behaviour of the enzyme, LmGMPR also appears to be highly regulated by ATP and GTP in the environment. Previous reports on GMP reductases from crude lysate preparations, including that of *L. donovani*, claimed that GTP had an activational effect on the enzyme (Renart et al. 1976; Spector et al. 1979; Spector et al. 1982). Purified His_6/S-LmGMPR did not appear to be affected by either GTP or ATP. However, untagged enzyme showed a 400% increase in reaction velocity by GTP at sub-K_m GMP concentrations and a decrease in $S_{0.5}$. At saturating GMP concentrations, GTP no longer exerted an effect on reaction rate indicating that the enzymes affinity for GMP is enhanced by GTP but not the overall V_{max}.

The presence of CBS domains in the LmGMPR suggests that there may have been a need for additional regulation of this enzyme within *Leishmania*. CBS domains have been implicated in binding of adenosine derivatives and in regulatory activities (Scott et al. 2004). As a result, ATP was investigated as a potential effector molecule. LmGMPR appears to display enhanced cooperative activity in the presence of ATP with relation to GMP concentration. At concentrations of GMP near the K_m ATP exerts a potent inhibitory effect. Under saturating GMP conditions the presence of ATP results in a 28 % lower apparent V_{max} and a ~2-fold increase in $S_{0.5}$.

Conceivably, ATP binding to LmGMPR may alter the enzymatic structure to cause a decrease in the affinity of LmGMPR for GMP and a subsequent decline in V_{max}. The unknown mechanism underlying the enzymes non-hyperbolic nature maybe responsible for the steep sigmoidal curve generated in the presence of ATP. In contrast, the inhibitory effect of ATP with regard to NADPH concentration is linear and conforms to Michaelis-Menten kinetics. However, inhibition requires 10-fold the ATP

concentration used to elicit a response with regard to GMP. The $S_{0.5}$ value is increased by 5-fold in the presence of 250 μM ATP and the apparent V_{max} decreases by 20%. This indicates that ATP exerts its effect predominantly on the binding of GMP.

This reactivity to downstream effector molecules places LmGMPR in an important position within the regulation of the *Leishmania* purine metabolic pathway. As ATP builds within the glycosome LmGMPR-mediated formation of IMP is inhibited. In contrast, when sufficient GMP builds up there is a resulting increase in GTP pools within the cell and LmGMPR is activated to begin shunting GMP back to IMP (Fig. 33). Whether these compounds bind the CBS domains is unknown but since they are both

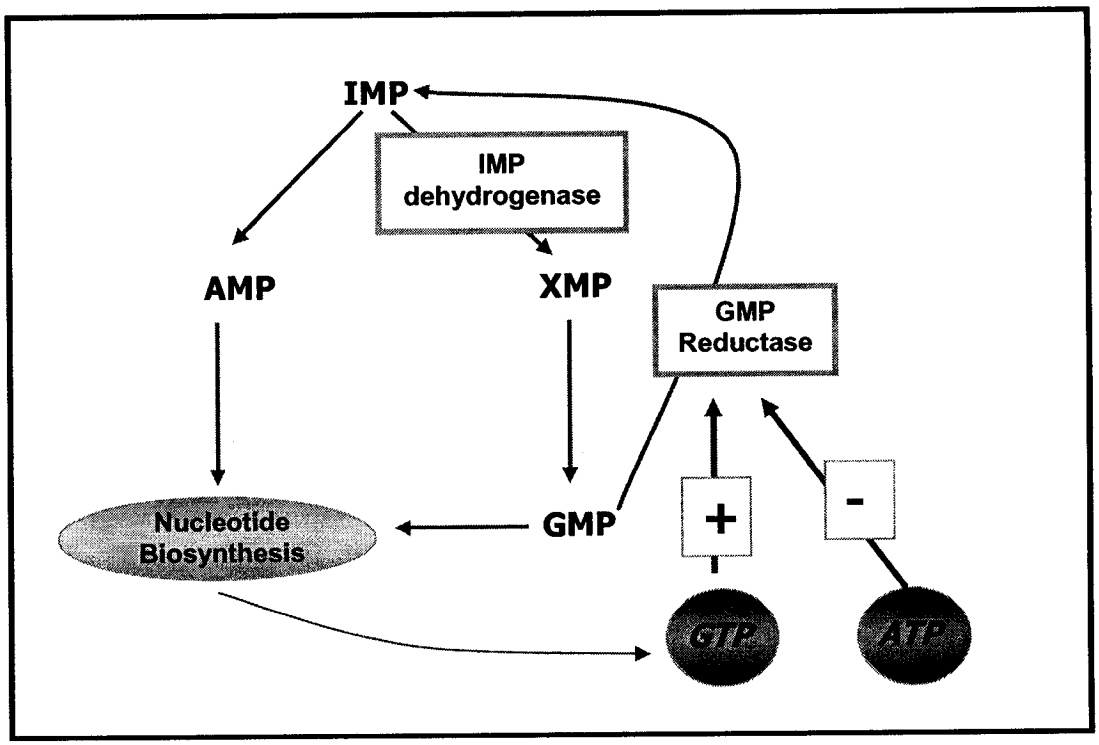

Figure 33. LmGMPR regulation by ATP and GTP. Increased GTP pools activate LmGMPR to convert GMP back to IMP whereas elevated ATP pools inhibit further shunting of GMP towards the formation of AMP.

heterocyclic structures that are very similar in composition it is not unlikely that they do exert their effect in the same allosteric location. Binding assays indicate that LmGMPR binds GTP and GMP stringently. Competition assays will determine whether GTP and GMP bind mutually exclusive sites on the enzyme. Elucidating the role of the LmGMPRs CBS domains will be crucial to understanding this parasite's purine salvage pathway.

While the CBS domains of LmGMPR may have been conserved throughout evolution for regulatory reasons it appears as if another relic of the ancestral enzyme has persisted as well. The MPA binding site is characteristic of IMPDHs. GMP reductases described thus far do not contain these motifs. Interestingly, the sequences of MPA binding sites have evolved across organisms. Bacterial IMPDHs tend to have a sequence with K_i values of ~10-20 μM for MPA whereas eukaryotic IMPDHs, containing the alternate MPA binding site sequence, tend to have K_is ~1000-fold lower (Verham et al. 1987; Carr et al. 1993; Sintchak et al. 1996; Zhou et al. 1997). The *Leishmania* IMPDH has a site identical to the eukaryotic sequence and this work has confirmed the finding of Wilson et al (1991) that the *Leishmania donovani* IMPDH has an EC_{50} of ~1 μM for MPA (Wilson et al. 1991). The LmGMPR has a MPA K_i of 20 μM and in *in vivo* dose response experiments with *L. donovani* the enzyme is not inhibited by MPA concentrations as high as 400 μM. This is likely due to the high K_i associated with this type of MPA binding site.

The preservation of this particular motif by LmGMPR is particularly interesting from an evolutionary standpoint because it sheds light on the origins of the LmGMPR. The retention of sequence similarity between prokaryotic and eukaryotic GMP reductases

suggests a common enzymic precursor, most likely the IMPDH (Becerra et al. 1998). However, the LmGMPR is substantially different from these enzymes. There are three scenarios for the drastic difference between the *Leishmania* GMPR and all other known GMPRs: the early divergence in the eukaryotic lineage and subsequent adaptation to a parasitic lifestyle drove the evolution of the common GMPR to form the unique *Leishmania* GMPR; the *Leishmania* GMPR may be a result of gene duplication of the IMPDH after the organisms divergence from the eukaryotic line; or selection pressure forced the *Leishmania* GMPR to retain certain attributes of the ancestral enzyme precursor. The latter is the most probable explanation for the divergence of the *Leishmania* GMPR.

The MPA binding site is crucial when examining each of these possibilities. The presence of an MPA binding site in LmGMPR negates the first theory. If the LmGMPR arose from an ancestral "common" GMPR the possibility of it developing an MPA binding site almost identical to those of bacteria would be highly unlikely. Gene duplication of the *Leishmania* IMPDH and evolution into the LmGMPR is argued against by the presence of an MPA binding site with similarity to bacterial IMPDHs. Recall that the *Leishmania* IMPDH has an MPA binding site identical to that of other eukaryotic IMPDHs. Therefore it is most probable that the selective pressure of being a parasitic organism drove the retention of certain ancestral IMPDH characteristics, such as the CBS domains, because of the benefits they conferred. The advantages of being able to tightly regulate a purine metabolic interconversion enzyme like LmGMPR would be critical for an organism incapable of synthesizing their own purines.

In addition to the interesting evolutionary information gleaned from the identification and characterization of the *Leishmania* GMP reductase, there are also potential implications for future chemotherapeutic directions. The reliance of this parasite on purine salvage makes it particularly vulnerable to treatment strategies targeting the enzymes within this pathway. Since GMP reductase is required to convert excess quantities of GMP back to IMP it may be an essential enzyme for *Leishmania* survival. The extensive regulation of this enzyme suggests that its activity is critical for the organism. However, future work with *L. donovani* knock-out parasites will confirm whether this is indeed the case. This work on the *Leishmania* GMP reductase has expanded our knowledge of the complex metabolic processes of these parasites and may, hopefully, assist the research community in the development of new anti-parasitic chemotherapies.

Literature Cited

Achterberg, V. and G. Gercken (1987). Cytotoxicity of ester and ether lysophospholipids on *Leishmania donovani* promastigotes. Mol Biochem Parasitol **23**(2): 117-22.

Alvar, J., C. Canavate, B. Gutierrez-Solar, M. Jimenez, F. Laguna, R. Lopez-Velez, R. Molina and J. Moreno (1997). *Leishmania* and human immunodeficiency virus coinfection: the first 10 years. Clin Microbiol Rev **10**(2): 298-319.

Andrews, S. C. and J. R. Guest (1988). Nucleotide sequence of the gene encoding the GMP reductase of *Escherichia coli* K12. Biochem J **255**(1): 35-43.

Antoine, J. C., E. Prina, C. Jouanne and P. Bongrand (1990). Parasitophorous vacuoles of *Leishmania amazonensis*-infected macrophages maintain an acidic pH. Infect Immun **58**(3): 779-87.

Aronow, B., K. Kaur, K. McCartan and B. Ullman (1987). Two high affinity nucleoside transporters in *Leishmania donovani*. Mol Biochem Parasitol **22**(1): 29-37.

Ashford, R. W. (2000). The leishmaniases as emerging and reemerging zoonoses. Int J Parasitol **30**(12-13): 1269-81.

Basselin, M., H. Denise, G. H. Coombs and M. P. Barrett (2002). Resistance to pentamidine in *Leishmania mexicana* involves exclusion of the drug from the mitochondrion. Antimicrob Agents Chemother **46**(12): 3731-8.

Bateman, A. (1997). The structure of a domain common to archaebacteria and the homocystinuria disease protein. Trends Biochem Sci **22**(1): 12-3.

Bates, P. A. (1993). Axenic culture of *Leishmania* amastigotes. Parasitol Today **9**(4): 143-6.

Beaty, P. S. and M. J. McInerney (1990). Nutritional Features of *Syntrophomonas wolfei*. Appl Environ Microbiol **56**(10): 3223-3224.

Becerra, A. and A. Lazcano (1998). The role of gene duplication in the evolution of purine nucleotide salvage pathways. Orig Life Evol Biosph **28**(4-6): 539-53.

Becker, J. L. (1974). Purine metabolism pathways in *Drosophila* cells grown "in vitro": Phosphoribosyl transferase activities. Biochimie **56**(5): 779-81.

Berens, R. L., Krug, E.C., & Marr, J.J. (1995). Purine and pyrimidine metabolism. Biochemistry and Molecular Biology of Parasites. J. J. M. Marr, M. New York, Academic Press.

Berman, J. D. (1997). Human leishmaniasis: clinical, diagnostic, and chemotherapeutic developments in the last 10 years. Clin Infect Dis **24**(4): 684-703.

Berman, J. D., J. V. Gallalee and J. M. Best (1987). Sodium stibogluconate (Pentostam) inhibition of glucose catabolism via the glycolytic pathway, and fatty acid beta-oxidation in *Leishmania mexicana* amastigotes. Biochem Pharmacol **36**(2): 197-201.

Berman, J. D. and F. A. Neva (1981). Effect of temperature on multiplication of *Leishmania* amastigotes within human monocyte-derived macrophages in vitro. Am J Trop Med Hyg **30**(2): 318-21.

Bernier, R., S. J. Turco, M. Olivier and M. Tremblay (1995). Activation of human immunodeficiency virus type 1 in monocytoid cells by the protozoan parasite *Leishmania donovani*. J Virol **69**(11): 7282-5.

Bissuel, F., L. Cotte, M. Rabodonirina, P. Rougier, M. A. Piens and C. Trepo (1994). Paromomycin: an effective treatment for cryptosporidial diarrhea in patients with AIDS. Clin Infect Dis **18**(3): 447-9.

Bontemps, F., L. Hue and H. G. Hers (1978). Phosphorylation of glucose in isolated rat hepatocytes. Sigmoidal kinetics explained by the activity of glucokinase alone. Biochem J **174**(2): 603-11.

Bork, P., J. Gellerich, H. Groth, R. Hooft and F. Martin (1995). Divergent evolution of a beta/alpha-barrel subclass: detection of numerous phosphate-binding sites by motif search. Protein Sci **4**(2): 268-74.

Bowne, S. J., L. S. Sullivan, S. H. Blanton, C. L. Cepko, S. Blackshaw, D. G. Birch, D. Hughbanks-Wheaton, J. R. Heckenlively and S. P. Daiger (2002). Mutations in the inosine monophosphate dehydrogenase 1 gene (IMPDH1) cause the RP10 form of autosomal dominant retinitis pigmentosa. Hum Mol Genet **11**(5): 559-68.

Brajtburg, J. and J. Bolard (1996). Carrier effects on biological activity of amphotericin B. Clin Microbiol Rev **9**(4): 512-31.

Bucheton, B., L. Abel, S. El-Safi, M. M. Kheir, S. Pavek, A. Lemainque and A. J. Dessein (2003). A major susceptibility locus on chromosome 22q12 plays a critical role in the control of kala-azar. Am J Hum Genet **73**(5): 1052-60.

Carr, S. F., E. Papp, J. C. Wu and Y. Natsumeda (1993). Characterization of human type I and type II IMP dehydrogenases. J Biol Chem **268**(36): 27286-90.

Chang, K.-P., Fong, D., & Bray, R.S. (1985). Biology of *Leishmania* and leishmaniasis. Leishmaniasis. K.-P. Chang, & Bray, R.S. New York, Elsevier Science Publishing Company Inc.

Chehimi, J., S. E. Starr, I. Frank, A. D'Andrea, X. Ma, R. R. MacGregor, J. Sennelier and G. Trinchieri (1994). Impaired interleukin 12 production in human immunodeficiency virus-infected patients. J Exp Med **179**(4): 1361-6.

Chenna, R., H. Sugawara, T. Koike, R. Lopez, T. J. Gibson, D. G. Higgins and J. D. Thompson (2003). Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Res **31**(13): 3497-500.

Clerici, M. and G. M. Shearer (1993). A TH1-->TH2 switch is a critical step in the etiology of HIV infection. Immunol Today **14**(3): 107-11.

Cohn, C. S. and M. Gottlieb (1997). The acquisition of purines by trypanosomatids. Parasitol Today **13**(6): 231-5.

Collart, F. R. and E. Huberman (1990). Expression of IMP dehydrogenase in differentiating HL-60 cells. Blood **75**(3): 570-6.

Convit, J., M. E. Pinardi and A. J. Rondon (1972). Diffuse cutaneous leishmaniasis: a disease due to an immunological defect of the host. Trans R Soc Trop Med Hyg **66**(4): 603-10.

Coombs, G. H., J. A. Craft and D. T. Hart (1982). A comparative study of *Leishmania mexicana* amastigotes and promastigotes. Enzyme activities and subcellular locations. Mol Biochem Parasitol **5**(3): 199-211.

Craig, S. P., 3rd, L. Yuan, D. A. Kuntz, J. H. McKerrow and C. C. Wang (1991). High level expression in *Escherichia coli* of soluble, enzymatically active schistosomal hypoxanthine/guanine phosphoribosyltransferase and trypanosomal ornithine decarboxylase. Proc Natl Acad Sci U S A **88**(6): 2500-4.

Croft, S. L. (2001). Monitoring drug resistance in leishmaniasis. Trop Med Int Health **6**(11): 899-905.

Croft, S. L. and R. P. Brazil (1982). Effect of pentamidine isethionate on the ultrastructure and morphology of *Leishmania mexicana amazonensis* in vitro. Ann Trop Med Parasitol **76**(1): 37-43.

Croft, S. L., S. Sundar and A. H. Fairlamb (2006). Drug resistance in leishmaniasis. Clin Microbiol Rev **19**(1): 111-26.

Croft, S. L. and V. Yardley (2002). Chemotherapy of leishmaniasis. Curr Pharm Des **8**(4): 319-42.

Dacks, J. B. and W. F. Doolittle (2001). Reconstructing/deconstructing the earliest eukaryotes: how comparative genomics can help. Cell **107**(4): 419-25.

Datta, A. K., D. Bhaumik and R. Chatterjee (1987). Isolation and characterization of adenosine kinase from *Leishmania donovani*. J Biol Chem **262**(12): 5515-21.

Davies, M. J., A. M. Ross and W. E. Gutteridge (1983). The enzymes of purine salvage in *Trypanosoma cruzi, Trypanosoma brucei* and *Leishmania mexicana*. Parasitology **87 (Pt 2)**: 211-7.

de Gorgolas, M. and M. A. Miles (1994). Visceral leishmaniasis and AIDS. Nature **372**(6508): 734.

Deng, Y., Z. Wang, K. Ying, S. Gu, C. Ji, Y. Huang, X. Gu, Y. Wang, Y. Xu, Y. Li, Y. Xie and Y. Mao (2002). NADPH-dependent GMP reductase isoenzyme of human (GMPR2). Expression, purification, and kinetic properties. Int J Biochem Cell Biol **34**(9): 1035-50.

Desjeux, P. (1999). Global control and *Leishmania* HIV co-infection. Clin Dermatol **17**(3): 317-25.

Desjeux, P. (2001). The increase in risk factors for leishmaniasis worldwide. Trans R Soc Trop Med Hyg **95**(3): 239-43.

Digits, J. A. and L. Hedstrom (1999). Kinetic mechanism of *Tritrichomonas foetus* inosine 5'-monophosphate dehydrogenase. Biochemistry **38**(8): 2295-306.

Digits, J. A. and L. Hedstrom (1999). Species-specific inhibition of inosine 5'-monophosphate dehydrogenase by mycophenolic acid. Biochemistry **38**(46): 15388-97.

Dwyer, D. M. and M. Gottlieb (1984). Surface membrane localization of 3'- and 5'-nucleotidase activities in *Leishmania donovani* promastigotes. Mol Biochem Parasitol **10**(2): 139-50.

Eisenthal, R. and A. Cornish-Bowden (1974). The direct linear plot. A new graphical procedure for estimating enzyme kinetic parameters. Biochem J **139**(3): 715-20.

El-On, J., S. Halevy, M. H. Grunwald and L. Weinrauch (1992). Topical treatment of Old World cutaneous leishmaniasis caused by *Leishmania major*: a double-blind control study. J Am Acad Dermatol **27**(2 Pt 1): 227-31.

El-On, J., G. P. Jacobs, E. Witztum and C. L. Greenblatt (1984). Development of topical treatment for cutaneous leishmaniasis caused by *Leishmania major* in experimental animals. Antimicrob Agents Chemother **26**(5): 745-51.

Farber, G. K. and G. A. Petsko (1990). The evolution of alpha/beta barrel enzymes. Trends Biochem Sci **15**(6): 228-34.

Ferdinand, W. (1966). The interpretation of non-hyperbolic rate curves for two-substrate enzymes. A possible mechanism for phosphofructokinase. Biochem J **98**(1): 278-83.

Fleischmann, R. D., M. D. Adams, O. White, R. A. Clayton, E. F. Kirkness, A. R. Kerlavage, C. J. Bult, J. F. Tomb, B. A. Dougherty, J. M. Merrick and et al. (1995). Whole-genome random sequencing and assembly of *Haemophilus influenzae Rd.* Science **269**(5223): 496-512.

Gan, L., G. A. Petsko and L. Hedstrom (2002). Crystal structure of a ternary complex of *Tritrichomonas foetus* inosine 5'-monophosphate dehydrogenase: NAD+ orients the active site loop for catalysis. Biochemistry **41**(44): 13309-17.

Gibson, Q. H. (1970). The reaction of oxygen with hemoglobin and the kinetic basis of the effect of salt on binding of oxygen. J Biol Chem **245**(13): 3285-8.

Gill, S. C. and P. H. von Hippel (1989). Calculation of protein extinction coefficients from amino acid sequence data. Anal Biochem **182**(2): 319-26.

Glew, R. H., A. K. Saha, S. Das and A. T. Remaley (1988). Biochemistry of the *Leishmania* species. Microbiol Rev **52**(4): 412-32.

Gottlieb, M. (1989). The surface membrane 3'-nucleotidase/nuclease of trypanosomatid protozoa. Parasitol Today **5**(8): 257-60.

Gould, S. J., G. A. Keller, N. Hosken, J. Wilkinson and S. Subramani (1989). A conserved tripeptide sorts proteins to peroxisomes. J Cell Biol **108**(5): 1657-64.

Gourbal, B., N. Sonuc, H. Bhattacharjee, D. Legare, S. Sundar, M. Ouellette, B. P. Rosen and R. Mukhopadhyay (2004). Drug uptake and modulation of drug resistance in *Leishmania* by an aquaglyceroporin. J Biol Chem **279**(30): 31010-7.

Gupta, N., N. Goyal and A. K. Rastogi (2001). In vitro cultivation and characterization of axenic amastigotes of *Leishmania*. Trends Parasitol **17**(3): 150-3.

Handman, E. (1999). Cell biology of *Leishmania*. Adv Parasitol **44**: 1-39.

Handman, E. and J. M. Curtis (1982). *Leishmania tropica*: surface antigens of intracellular and flagellate forms. Exp Parasitol **54**(2): 243-9.

Hansen, B. D., J. Perez-Arbelo, J. F. Walkony and L. D. Hendricks (1982). The specificity of purine base and nucleoside uptake in promastigotes of *Leishmania braziliensis panamensis*. Parasitology **85 (Pt 2)**: 271-82.

Hitchings, G. H. (1982). The purine metabolism of protozoa. Adv Enzyme Regul **20**: 375-86.

Jackson, R. C., G. Weber and H. P. Morris (1975). IMP dehydrogenase, an enzyme linked with proliferation and malignancy. Nature **256**(5515): 331-3.

Janeway, C. A., Travers, P., Walport, M., Shlomchik, M. (2001). Immunobiology: The Immune System in Health and Disease. New York, Garland Publishing.

Jardim, A., S. E. Bergeson, S. Shih, N. Carter, R. W. Lucas, G. Merlin, P. J. Myler, K. Stuart and B. Ullman (1999). Xanthine phosphoribosyltransferase from *Leishmania donovani*. Molecular cloning, biochemical characterization, and genetic analysis. J Biol Chem **274**(48): 34403-10.

Jha, T. K., P. Olliaro, C. P. Thakur, T. P. Kanyok, B. L. Singhania, I. J. Singh, N. K. Singh, S. Akhoury and S. Jha (1998). Randomised controlled trial of aminosidine (paromomycin) versus sodium stibogluconate for treating visceral leishmaniasis in North Bihar, India. Bmj **316**(7139): 1200-5.

Jha, T. K., S. Sundar, C. P. Thakur, P. Bachmann, J. Karbwang, C. Fischer, A. Voss and J. Berman (1999). Miltefosine, an oral agent, for the treatment of Indian visceral leishmaniasis. N Engl J Med **341**(24): 1795-800.

Kakkar, T., H. Boxenbaum and M. Mayersohn (1999). Estimation of K_i in a competitive enzyme-inhibition model: comparisons among three methods of data analysis. Drug Metab Dispos **27**(6): 756-62.

Kanno, H., I. Y. Huang, Y. W. Kan and A. Yoshida (1989). Two structural genes on different chromosomes are required for encoding the major subunit of human red cell glucose-6-phosphate dehydrogenase. Cell **58**(3): 595-606.

Konigk, E. and B. Putfarken (1980). Stage-specific differences of a perhaps signal-transferring system in *Leishmania donovani*. Tropenmed Parasitol **31**(4): 421-4.

Kuhlencord, A., T. Maniera, H. Eibl and C. Unger (1992). Hexadecylphosphocholine: oral treatment of visceral leishmaniasis in mice. Antimicrob Agents Chemother **36**(8): 1630-4.

Levitzki, A. and D. E. Koshland, Jr. (1969). Negative cooperativity in regulatory enzymes. Proc Natl Acad Sci U S A **62**(4): 1121-8.

Liew, F. Y. and C. A. O'Donnell (1993). Immunology of leishmaniasis. Adv Parasitol **32**: 161-259.

Looker, D. L., R. L. Berens and J. J. Marr (1983). Purine metabolism in *Leishmania donovani* amastigotes and promastigotes. Mol Biochem Parasitol **9**(1): 15-28.

Maarouf, M., M. T. Adeline, M. Solignac, D. Vautrin and M. Robert-Gero (1998). Development and characterization of paromomycin-resistant *Leishmania donovani* promastigotes. Parasite **5**(2): 167-73.

Mackenzie, J. J. and L. B. Sorensen (1973). Guanosine 5'-phosphate reductase of human erythrocytes. Biochim Biophys Acta **327**(2): 282-94.

Mager, J. and B. Magasnik (1960). Guanosine 5'-phosphate reductase and its role in the interconversion of purine nucleotides. J Biol Chem **235**: 1474-8.

Marr, J. J., & Berens, R.L. (1985). Purine and pyrimidine metabolism in *Leishmania*. Leishmaniasis. K.-P. Chang, & Bray, R.S. New York, Elsevier Science Publishing Company Inc.

Marr, J. J., R. L. Berens and D. J. Nelson (1978). Purine metabolism in *Leishmania donovani* and *Leishmania braziliensis*. Biochim Biophys Acta **544**(2): 360-71.

Maslov, D. A., S. A. Podlipaev and J. Lukes (2001). Phylogeny of the kinetoplastida: taxonomic problems and insights into the evolution of parasitism. Mem Inst Oswaldo Cruz **96**(3): 397-402.

Mbongo, N., P. M. Loiseau, M. A. Billion and M. Robert-Gero (1998). Mechanism of amphotericin B resistance in *Leishmania donovani* promastigotes. Antimicrob Agents Chemother **42**(2): 352-7.

McInerney, M. J., M. P. Bryant, R. B. Hespell and J. W. Costerton (1981). *Syntrophomonas wolfei* gen. nov. sp. nov., an Anaerobic, Syntrophic, Fatty Acid-Oxidizing Bacterium. Appl Environ Microbiol **41**(4): 1029-1039.

Mele, T. S. and P. F. Halloran (2000). The use of mycophenolate mofetil in transplant recipients. Immunopharmacology **47**(2-3): 215-45.

Momeni, A. Z., M. R. Reiszadae and M. Aminjavaheri (2002). Treatment of cutaneous leishmaniasis with a combination of allopurinol and low-dose meglumine antimoniate. Int J Dermatol **41**(7): 441-3.

Mosier, D. and H. Sieburg (1994). Macrophage-tropic HIV: critical for AIDS pathogenesis? Immunol Today **15**(7): 332-9.

Mosser, D. M. and P. J. Edelson (1985). The mouse macrophage receptor for C3bi (CR3) is a major mechanism in the phagocytosis of *Leishmania* promastigotes. J Immunol **135**(4): 2785-9.

Murray, H. W. and S. Delph-Etienne (2000). Visceral leishmanicidal activity of hexadecylphosphocholine (miltefosine) in mice deficient in T cells and activated macrophage microbicidal mechanisms. J Infect Dis **181**(2): 795-9.

Murray, H. W., M. J. Oca, A. M. Granger and R. D. Schreiber (1989). Requirement for T cells and effect of lymphokines in successful chemotherapy for an intracellular infection. Experimental visceral leishmaniasis. J Clin Invest **83**(4): 1253-7.

Neet, K. E. (1996). Cooperativity in Enzyme Function: Equilibrium and Kinetic Aspects. Contemporary Enzyme Kinetics and Mechanism. D. L. Purich. San Diego, Academic Press: 133-182.

Nelson, D. L., & Cox, M.M. (2005). Lehninger Biochemistry 4th Edition. New York., W.H Freeman and Company.

Opperdoes, F. R. (1984). Localization of the initial steps in alkoxyphospholipid biosynthesis in glycosomes (microbodies) of *Trypanosoma brucei*. FEBS Lett **169**(1): 35-9.

Opperdoes, F. R. and P. Borst (1977). Localization of nine glycolytic enzymes in a microbody-like organelle in *Trypanosoma brucei*: the glycosome. FEBS Lett **80**(2): 360-4.

Pamba, H. O., B. B. Estambale, C. N. Chunge and L. Donno (1990). Comparative study of aminosidine, etophamide and nimorazole, alone or in combination, in the treatment of intestinal amoebiasis in Kenya. Eur J Clin Pharmacol **39**(4): 353-7.

Pan, A. A. (1984). *Leishmania mexicana*: serial cultivation of intracellular stages in a cell-free medium. Exp Parasitol **58**(1): 72-80.

Pfaller, M. A. and J. J. Marr (1974). Antileishmanial effect of allopurinol. Antimicrob Agents Chemother **5**(5): 469-72.

Pimenta, P. F., E. M. Saraiva, E. Rowton, G. B. Modi, L. A. Garraway, S. M. Beverley, S. J. Turco and D. L. Sacks (1994). Evidence that the vectorial competence of phlebotomine sand flies for different species of *Leishmania* is controlled by structural polymorphisms in the surface lipophosphoglycan. Proc Natl Acad Sci U S A **91**(19): 9155-9.

Pimenta, P. F., S. J. Turco, M. J. McConville, P. G. Lawyer, P. V. Perkins and D. L. Sacks (1992). Stage-specific adhesion of *Leishmania* promastigotes to the sandfly midgut. Science **256**(5065): 1812-5.

Plagemann, P. G., R. M. Wohlhueter and J. Erbe (1981). Facilitated transport of inosine and uridine in cultured mammalian cells is independent of nucleoside phosphorylases. Biochim Biophys Acta **640**(2): 448-62.

Plagemann, P. G., R. M. Wohlhueter and M. Kraupp (1985). Adenosine uptake, transport, and metabolism in human erythrocytes. J Cell Physiol **125**(2): 330-6.

Puig, L. and R. Pradinaud (2003). *Leishmania* and HIV co-infection: dermatological manifestations. Ann Trop Med Parasitol **97 Suppl 1**: 107-14.

Reardon, D. and G. K. Farber (1995). The structure and evolution of alpha/beta barrel proteins. Faseb J **9**(7): 497-503.

Reiner, S. L. and R. M. Locksley (1995). The regulation of immunity to *Leishmania major*. Annu Rev Immunol **13**: 151-77.

Renart, M. F., J. Renart, M. A. Sillero and A. Sillero (1976). Guanosine monophosphate reductase from *Artemia salina*: Inhibition by xanthosine monophosphate and activation by diguanosine tetraphosphate. Biochemistry **15**(23): 4962-6.

Rosenthal, L. A., F. S. Sutterwala, M. E. Kehrli and D. M. Mosser (1996). *Leishmania major*-human macrophage interactions: cooperation between Mac-1 (CD11b/CD18) and complement receptor type 1 (CD35) in promastigote adhesion. Infect Immun **64**(6): 2206-15.

Runge, M. H., R. Andreesen, A. Pfleiderer and P. G. Munder (1980). Destruction of human solid tumors by alkyl lysophospholipids. J Natl Cancer Inst **64**(6): 1301-6.

Salvatore, D., T. Bartha and P. R. Larsen (1998). The guanosine monophosphate reductase gene is conserved in rats and its expression increases rapidly in brown adipose tissue during cold exposure. J Biol Chem **273**(47): 31092-6.

Schmidt-Rose, T. and T. J. Jentsch (1997). Reconstitution of functional voltage-gated chloride channels from complementary fragments of CLC-1. J Biol Chem **272**(33): 20515-21.

Scott, J. W., S. A. Hawley, K. A. Green, M. Anis, G. Stewart, G. A. Scullion, D. G. Norman and D. G. Hardie (2004). CBS domains form energy-sensing modules whose binding of adenosine ligands is disrupted by disease mutations. J Clin Invest **113**(2): 274-84.

Seaman, J., A. J. Mercer and E. Sondorp (1996). The epidemic of visceral leishmaniasis in western Upper Nile, southern Sudan: course and impact from 1984 to 1994. Int J Epidemiol **25**(4): 862-71.

Shaked-Mishan, P., N. Ulrich, M. Ephros and D. Zilberstein (2001). Novel Intracellular SbV reducing activity correlates with antimony susceptibility in *Leishmania donovani*. J Biol Chem **276**(6): 3971-6.

Shih, S., H. Y. Hwang, D. Carter, P. Stenberg and B. Ullman (1998). Localization and targeting of the *Leishmania donovani* hypoxanthine-guanine phosphoribosyltransferase to the glycosome. J Biol Chem **273**(3): 1534-41.

Sintchak, M. D., M. A. Fleming, O. Futer, S. A. Raybuck, S. P. Chambers, P. R. Caron, M. A. Murcko and K. P. Wilson (1996). Structure and mechanism of inosine monophosphate dehydrogenase in complex with the immunosuppressant mycophenolic acid. Cell **85**(6): 921-30.

Soto, J., P. Buffet, M. Grogl and J. Berman (1994). Successful treatment of Colombian cutaneous leishmaniasis with four injections of pentamidine. Am J Trop Med Hyg **50**(1): 107-11.

Spector, T. and T. E. Jones (1982). Guanosine 5'-monophosphate reductase from *Leishmania donovani*. A possible chemotherapeutic target. Biochem Pharmacol **31**(23): 3891-7.

Spector, T., T. E. Jones and R. L. Miller (1979). Reaction mechanism and specificity of human GMP reductase. Substrates, inhibitors, activators, and inactivators. J Biol Chem **254**(7): 2308-15.

Stephens, R. W. and V. K. Whittaker (1973). Calf thymus GMP reductase: control by XMP. Biochem Biophys Res Commun **53**(3): 975-81.

Sundar, S., F. Rosenkaimer, M. K. Makharia, A. K. Goyal, A. K. Mandal, A. Voss, P. Hilgard and H. W. Murray (1998). Trial of oral miltefosine for visceral leishmaniasis. Lancet **352**(9143): 1821-3.

Sundar, S., B. B. Thakur, A. K. Tandon, N. R. Agrawal, C. P. Mishra, T. M. Mahapatra and V. P. Singh (1994). Clinicoepidemiological study of drug resistance in Indian kala-azar. Bmj **308**(6924): 307.

Swinkels, B. W., S. J. Gould, A. G. Bodnar, R. A. Rachubinski and S. Subramani (1991). A novel, cleavable peroxisomal targeting signal at the amino-terminus of the rat 3-ketoacyl-CoA thiolase. Embo J **10**(11): 3255-62.

Thakur, C. P. (2000). Socio-economics of visceral leishmaniasis in Bihar (India). Trans R Soc Trop Med Hyg **94**(2): 156-7.

Turco, S. J. and D. L. Sacks (1991). Expression of a stage-specific lipophosphoglycan in *Leishmania major* amastigotes. Mol Biochem Parasitol **45**(1): 91-9.

Umejiego, N. N., C. Li, T. Riera, L. Hedstrom and B. Striepen (2004). *Cryptosporidium parvum* IMP dehydrogenase: identification of functional, structural, and dynamic properties that can be exploited for drug design. J Biol Chem **279**(39): 40320-7.

Vercesi, A. E. and R. Docampo (1992). Ca2+ transport by digitonin-permeabilized *Leishmania donovani*. Effects of Ca2+, pentamidine and WR-6026 on mitochondrial membrane potential in situ. Biochem J **284 (Pt 2)**: 463-7.

Verham, R., T. D. Meek, L. Hedstrom and C. C. Wang (1987). Purification, characterization, and kinetic analysis of inosine 5'-monophosphate dehydrogenase of *Tritrichomonas foetus*. Mol Biochem Parasitol **24**(1): 1-12.

WHO (2000). Fact sheet 116: The leishmaniases and *Leishmania*/HIV co-infections.

WHO (2002). Strategic direction for research.

WHO (2004). Disease Watch- Focus: Leishmaniasis.

Wiemer, E. A., I. J. L, J. van Roy, R. J. Wanders and F. R. Opperdoes (1996). Identification of 2-enoyl coenzyme A hydratase and NADP(+)-dependent 3-hydroxyacyl-CoA dehydrogenase activity in glycosomes of procyclic *Trypanosoma brucei*. Mol Biochem Parasitol **82**(1): 107-11.

Wilson, K., F. R. Collart, E. Huberman, J. R. Stringer and B. Ullman (1991). Amplification and molecular cloning of the IMP dehydrogenase gene of *Leishmania donovani*. J Biol Chem **266**(3): 1665-71.

Zarella-Boitz, J. M., N. Rager, A. Jardim and B. Ullman (2004). Subcellular localization of adenine and xanthine phosphoribosyltransferases in *Leishmania donovani*. Mol Biochem Parasitol **134**(1): 43-51.

Zhang, J., W. Zhang, D. Zou, G. Chen, T. Wan, M. Zhang and X. Cao (2003). Cloning and functional characterization of GMPR2, a novel human guanosine monophosphate reductase, which promotes the monocytic differentiation of HL-60 leukemia cells. J Cancer Res Clin Oncol **129**(2): 76-83.

Zhang, R., G. Evans, F. J. Rotella, E. M. Westbrook, D. Beno, E. Huberman, A. Joachimiak and F. R. Collart (1999). Characteristics and crystal structure of bacterial inosine-5'-monophosphate dehydrogenase. Biochemistry **38**(15): 4691-700.

Zhou, X., M. Cahoon, P. Rosa and L. Hedstrom (1997). Expression, purification, and characterization of inosine 5'-monophosphate dehydrogenase from *Borrelia burgdorferi*. J Biol Chem **272**(35): 21977-81.

Zilberstein, D. and M. Shapira (1994). The role of pH and temperature in the development of *Leishmania* parasites. Annu Rev Microbiol **48**: 449-70.