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Independent phosphoglycerate mutase and pyruvate phosphate dikinase: potential targets for drug discovery in filariasis

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Table of contents

Abbreviations

2-PG:	2-phosphoglycerate
3-PG:	3-phosphoglycerate
aa:	amino-acid
ABTS:	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
ABZ:	Albendazole
ADP:	Adenosine Diphosphate
AMP:	Adenosine Monophosphate
ATP:	Adenosine Triphosphate
BSA:	Bovine serum albumine
CATT:	card agglutination test for trypanosomes
cDNA:	coding deoxyribonucleic acid
CIATT:	Card Indirect Agglutination Test for Trypanosomes
DALY:	Disability Adjusted Life Years
DEC:	Diethylcarbamazine
DEG	Database of Essential Genes
DFMO:	α-difluoromethylornithine
DNA:	Desoxyribonucleotid Acid
dPGAM:	dependent Phosphoglycerate Mutase
dPGM:	Dependent PhosphoGlycerate Mutase
dsRNA:	double-stranded DNA
DTT:	Dithiothreitol
EDTA:	Ethylendiamintetraacetic acid
ELISA:	Enzymes Linked Immonosorbent Assay
ENO:	enolase
EST:	Expressed Sequence Tag
FBA I:	fructose-bisphosphate aldolase Class I
FBA II:	fructose-bisphosphate aldolase class II
GABA:	Gamma-aminobutyric acid
GAP:	glyceraldehydes 3-phosphate dehydrogenase
GLK:	glucokinase

GPI:	glucose-6-phosphate-isomerase
h:	hour(s)
HAPT:	High Affinity Pentamidine Transporter
HEX:	hexokinase
HRP:	Horseradish Peroxidase
iPGAM:	independent Phosphoglycerate Mutase
iPGM:	Independent Phosphoglycerate Mutase
IPTG:	Isopropyl-1-β-D-thiogalactopyranoside
IVM:	Ivermectin
kb:	kilobase
kDa:	kilo-Dalton
LAPT:	Low-Affinity Pentamidine Transporter
LB:	Luria Bertani
LF:	Lymphatic Filariasis
MBP:	Maltose Binding Protein
MDA:	Mass Drug Administration
min:	minute(s)
MOPS:	3-(N-morpholino) propanesulfonic acid
NAD:	Nicotinamide adenine dinucleotide
NADH:	Nicotinamide adenine dinucleotide, hydrogen
NCBI:	National Center for Biotechnology Information
NEB:	New England Biolabs
NIAID:	National Institute of Allergy and Infectious Diseases
NiNTA:	Nickel chelating agarose resin (QIAGEN)
ORF:	Open Reading Frame
PBS:	Phosphate Buffer Saline
PCR:	Polymerase Chain Reaction
PEG:	Poly Ethylene Glycol
PEP:	phosphoenolpyruvate
PFK:	6-phosphofructokinase

PFP:	pyrophosphate::fructose 6-phosphate phosphotransferase
Pfu:	Phage forming unit
PGK:	phosphoglycerokinase
Pi:	inorganic phosphate
p*I:*	isoelectric point
PK:	pyruvate kinase
PPDK:	pyruvate phosphate dikinase
PPi:	pyrophosphate
RNA:	Ribonucleic acid
RNAi:	Ribonucleic acid interference
rpm:	rotation per minute
RT:	Room Temperature
SDS:	Sodium Dodecyl Sulfate
SDS-PAGE:	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
Sec:	second(s)
TBS:	Tris Buffer Saline
TBST:	Tris Buffer Saline Tween-20
TCA:	tricarboxylic acid cycle
TDR:	Special Programme for Reseach and Training in Tropical Diseases
TE:	Tris EDTA
TPI:	triose phosphate isomerase
*w*Bm:	*Wolbachia* from *Brugia malayi*
WHO:	World Health Organization
YCB:	Yeast Base Carbon

General Introduction

Neglected diseases and filarial parasites

The majority of neglected diseases in humans are caused by parasites. They are mainly found in tropical countries and affect more than one billion people, normally in the poorest regions of the world and are in fact a leading cause of poverty. Some of the infections are lethal while many are often disabling, disfiguring, and stigmatizing. In most cases, treatment options are inadequate or do not exist, and there is insufficient drug-market potential to attract pharmaceutical manufacturers. Examples of neglected diseases caused by protozoan parasites are malaria, leishmaniasis, and trypanosomiasis (Chagas disease and sleeping sickness). Parasitic worms (helminths) are responsible for schistosomiasis and filariasis (lymphatic and onchocerciasis). The World Health Organization (WHO) and the Special Programme for Research and Training in Tropical Diseases (TDR) have had a long standing interest in infectious diseases in the developing world and have been involved with academic and industrial partners in developing methods to improve prevention, detection and treatment. For more than 25 years, New England Biolabs (NEB) has been performing basic research on human filariasis. Dr. Donald Comb, the founder of NEB, recognized the need for basic research on these most neglected diseases in order to discover new methods for parasite control, and thereby improve the quality of life in areas where filarial infections exist.

Filariasis refers to a group of human and animal infectious diseases caused by thread-like nematode parasites, 'filariae', of the Order Spirurida. The adult male and female worms live in vessels, body cavities or tissues of the vertebrate host (definitive host) and are viviparous, producing live young called microfilariae (first-stage larvae) which circulate in the blood or skin. The microfilariae are ingested by blood sucking insects (intermediate hosts) in which they undergo further development to the infective stage (third-stage larvae) before being transmitted to a new definitive host. Most filarial parasites are both host- and infection site-specific. Lymphatic filariasis (LF) and onchocerciasis are the most serious diseases of this group.

Lymphatic filariasis

LF was first described thousands of years ago in ancient Greek and Roman texts, and captured in an ancient Egyptian statue depicting the characteristic swellings of the limbs

commonly known as elephantiasis. Microfilaria and adult worms were only discovered, identified and linked to the disease in the 19th century (Cox, 2002). LF is caused by parasitic filarial nematodes that live in the lymphatic system of the human host. Several species are known to be the causative agents of the disease, including *Wuchereria bancrofti* (*W. bancrofti*), *Brugia malayi* (*B. malayi*) and *Brugia timori* (*B. timori*).

LF parasites are transmitted to humans by a large variety of mosquito vectors (*Anopheles*, *Culex*, *Aedes*, *Mansonia*). *W. bancrofti* is by far the most prevalent, responsible for ~90% of the cases in tropical and sub-tropical regions of the world (Figure 1). *B. malayi* is confined to South and Southeast Asia, while *B. timori* is only found in Timor and neighboring islands. LF, is after malaria, the second most important/prevalent vector-borne parasitic disease in the world with 120 million people infected and 1.3 billion at risk (Melrose, 2002). The disease is reported to be responsible for 5 million Disability Adjusted Life Years (DALYs) lost annually, ranking third among the TDR diseases in terms of DALYs, after malaria and TB. India and Africa together account for 85-90% of the estimated burden of disease in terms of DALYs (WHO, 2002).

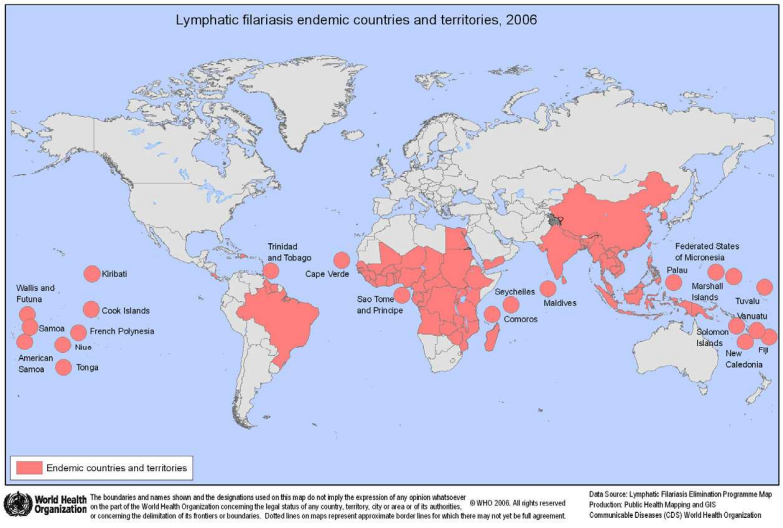

Figure 1: Distribution of lymphatic filariasis in the world.
(Source: WHO, 2006)

LF is considered one of the world's most disabling and disfiguring diseases, (Melrose, 2002; Leggat *et al.*, 2004) largely due to the gross swelling of the lower limbs or lymphoedema (Figure 2), as well as the genitalia in the case of hydrocoele. Acute inflammation of the lymph ducts and lymph nodes can also occur. *W. bancrofti* is only found in humans and will not infect small laboratory animals, so it is extremely difficult to obtain parasite material for study in the

lab. As a result, research on this parasite has been severely restricted. In contrast, natural infections of *B. malayi* have been reported in a number of animals and the worms will develop to maturity in the Mongolian gerbil (*Meriones unguiculatus*), thereby enabling scientists to maintain the life cycle in the laboratory. The *B. malayi*/gerbil system is the model system of choice used to study many aspects of filarial infection and has provided the material necessary for molecular, biochemical, immunological and genomic studies on the parasite. The genome of *B. malayi* was recently sequenced (Ghedin *et al.*, 2007), representing the first parasitic helminth genome to be completed, and has provided an unprecedented wealth of information for further studies on filariasis.

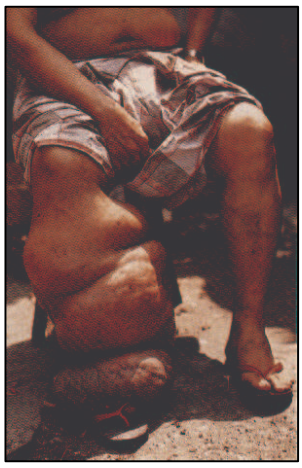

Figure 2: Elephantiasis resulting from an advanced stage of infection by filarial parasites.
(Source: Filarial Genome Network)

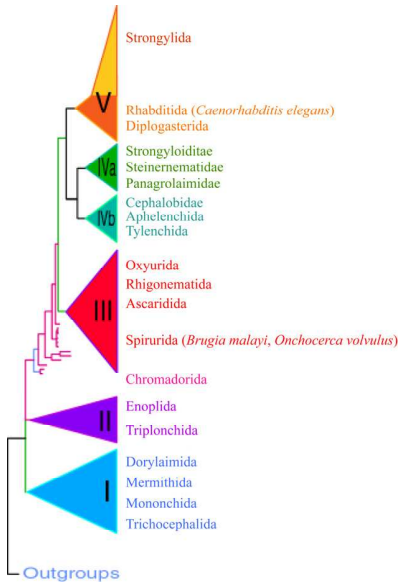

Figure 3: The phylogenetic structure of the Nematoda revealed by analysis of full-length small subunit (SSU) rDNA sequences. (adapted from (Dorris *et al.*, 1999))

The morphology of *B. malayi* worms is very similar to those of *W. bancrofti*. Both species belong to Clade III in the Order Spirurida (Figure 3) (Dorris *et al.*, 1999). Adult female *B. malayi* are larger than the males, varying in size from 43.5-55.0 x 0.13-0.17 mm, while the males are 13.5-23.3 x 0.07-0.08 mm (Sasa, 1976). They can live for more than a decade in the host during which time the female worm is constantly producing microfilariae or first-stage larvae. The microfilariae (170-260 x 5-6 μm) have a lifespan of many months and possess nocturnal periodicity, meaning that they circulate in the blood at night and sequester in tissues during the day. The most common mosquito vectors of Malayan filariasis are night-biting species of *Mansonia*. Following ingestion by the vector, the parasite undergoes 2 molts over the course of approximately 10 days to reach the L3 infective larvae (1.3-1.7 mm in length) stage capable of transmitting infection to another human (Muller and Baker, 1990). In a human, another 2 molts take place to produce adult worms which will mate and produce micofilariae (Figure 4).

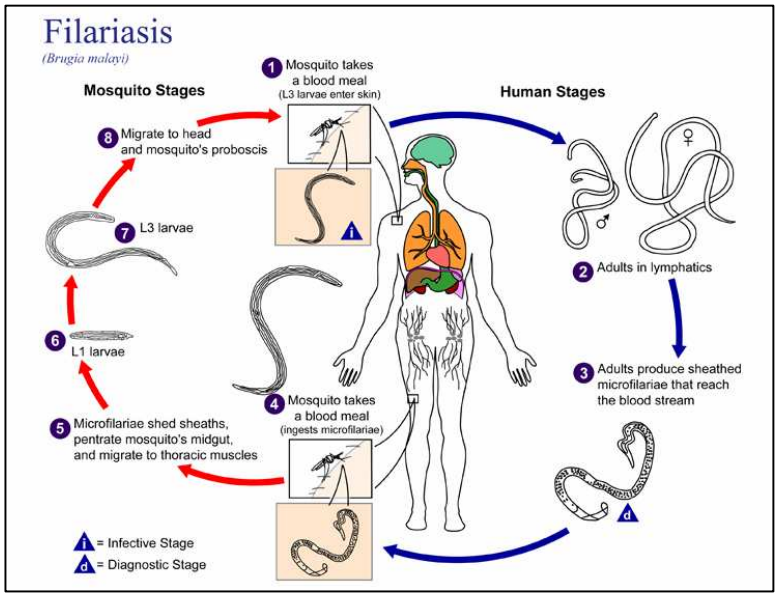

Figure 4: Life cycle of the filarial nematode *Brugia malayi*.
(Source: http://www.dpd.cdc.gov/dpdx)

Diagnosis of LF infection in the absence of gross swelling is challenging and the most commonly used method in the field involves detection of microfilariae in blood drawn at night and determination of the species by Giemsa staining of blood smears (Figure 5). More recently,

polymerase chain reaction (PCR), antigen detection, X-rays and ultrasonography have been used in the better equipped laboratories in endemic countries (Leggat *et al.*, 2004).

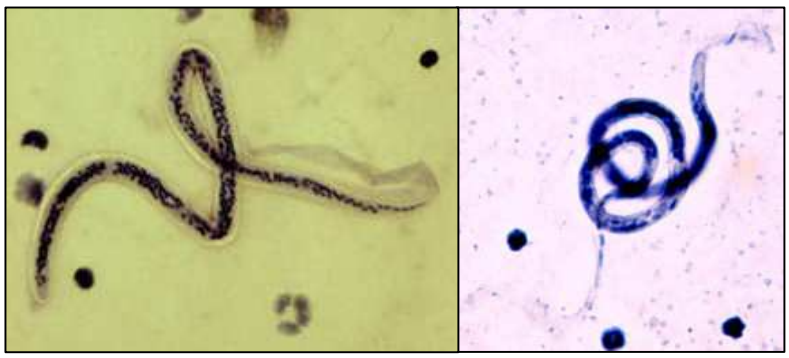

Figure 5: Causatives agents of lymphatic filariasis.
Blood smears containing hematoxylin-stained microfilariae of *Wuchereria bancrofti* (left panel) and Giemsa-stained *Brugia malayi* (right panel). (Source: wikipedia.org)

Onchocerciasis

Onchocerciasis, river blindness or *craw-craw* disease was first described during the 19th century when the filarial parasite was identified in skin snips from a patient (Cox, 2002). Currently, approximately 18-40 million people are infected and the disease is endemic in more than 36 countries in Africa, and part of the Arabic peninsula and Central and South America (WHO, 2006) (Figure 6). It is the second leading cause of infectious blindness worldwide and results in a huge economic burden on the society as a result of severe disability (Burnham, 1998; Hoerauf *et al.*, 2003). According to a 2002 WHO report, its global burden is 987,000 DALYs, with the severe pruritis alone accounting for 60% of the DALYs.

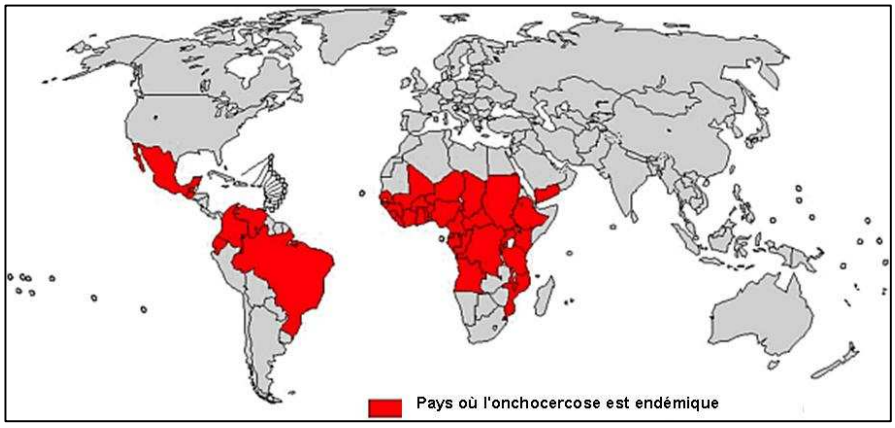

Figure 6: Distribution of onchocerciasis.
Endemic countries are indicated in red. (Source: WHO)

The causative agent is the filarial nematode *Onchocerca volvulus* (*O. volvulus*), transmitted by flies of the genus *Simulium*, known as blackflies or buffalo gnats (Figure 7). Blackflies are confined to streams and rivers for breeding, as their larvae need to attach to vegetation or rocks near well oxygenated, flowing water. Therefore the disease is found in these areas and hence the name river blindness. *O. volvulus* also belongs to Clade III in the Order Spirurida (Figure 3) (Dorris *et al.*, 1999) and shares quite a number of characteristics with the LF parasites.

Figure 7: left panel: Black fly (*Simulium spp.*) biting human host. Right panel: Adult *O. volvulus* worm.
(Source: www.stanford.edu)

The adults have a lifespan of 12-15 years and the female is viviparous, measuring up to 400 x 0.3 mm, while the male is much smaller reaching 30 x 0.2 mm in size (Muller and Baker, 1990). They live in subcutaneous tissues on the torso, head and near joints where they can form tangled masses inside fibrous nodules (Figure 7). The microfilariae circulate in the skin and eyes, and measure 280-330 x 6-9 μm (Muller and Baker, 1990). Once they have been ingested by *Simulium* blackflies, the microfilariae molt twice to become infective, third stage larvae and increase in size to 440-700 x 19 μm (Figure 8). *O. volvulus* infection is characterized by scaly, itchy, nodular skin and blindness in the worst cases (Figure 9). Microfilariae cause blindness as a result of an inflammatory reaction when they die in the cornea.

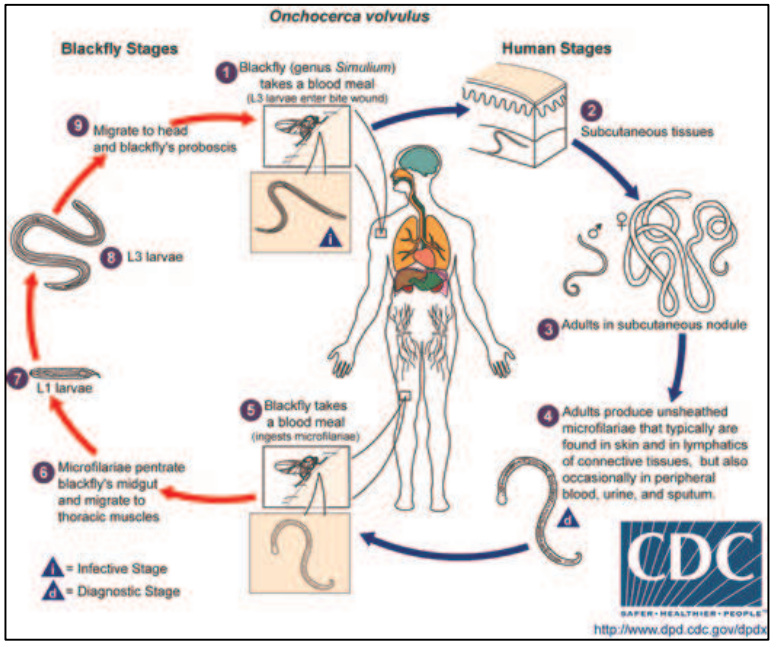

Figure 8: Life cycle of the filarial nematode *O. volvulus*.
(Source: http://www.dpd.cdc.gov/dpdx)

Figure 9: Individuals infected with *O. volvulus*.
Ocular lesions (left panel), nodules and skin involvement (right panel) are shown. (Source: www.icp.ucl.ac.be)

Diagnosis of *O. volvulus* infection often relies on palpation of the nodules and detection of microfilariae in the eyes using a slit lamp during physical exam. The most commonly used (and painful) diagnostic procedure involves isolation of microfilariae from a skin snip following incubation in a saline solution for 30 minutes. The highly motile parasites which emerge can easily be visualized under a microscope. A Mazzotti test can be performed in some cases using a dose of 50 mg diethylcarbamazine (DEC), which will provoke an intense itching and erythema

caused by allergic reactions to dying microfilariae. Various immunological tests and PCR are also being employed in more developed laboratories in certain countries (Enk, 2006).

Treatment

Very few drugs are available to treat human filariasis. Three are currently in use namely ivermectin (IVM), albendazole (ABZ) and diethylcarbamazine (DEC), and were discovered through animal health research and then adapted for use in humans. Over the past 30 years WHO/TDR and various government agencies have collaborated with drug companies to try to control and eliminate these diseases through mass drug administration (MDA) (Woods *et al.*, 2007) and vector control. Merck and Co. Inc was the first company to participate in the 80s and generously donated Mectizan (IVM) to various countries in Africa for the treatment of river blindness. More recently, GlaxoSmithKline joined the effort with donations of ABZ. These drugs are being administered in annual MDA programs either singly (IVM for onchocerciasis) or in combination for LF (ABZ plus IVM or IVM plus DEC in areas free of onchocerciasis) (Townson *et al.*, 2007).

Ivermectin

Ivermectin (Figure 10) is a macrocyclic lactone discovered in the 1970s by scientists at Merck. IVM was isolated from the bacterium *Streptomyces avermitilis* and is to date the most potent and broadest spectrum drug against nematodes (Woods *et al.*, 2007). It binds with high affinity to invertebrate glutamate-gated chloride ion channels resulting in hyperpolarization, inhibition of the pharyngeal pumping, paralysis, inhibition of the reproduction and fairly rapid death of the parasite (Prichard, 2007). IVM also is believed to act as an agonist of the neurotransmitter gamma-aminobutyric acid (GABA), thereby disrupting GABA-mediated central nervous system (CNS) neurosynaptic transmission (Markell *et al.*, 1992).

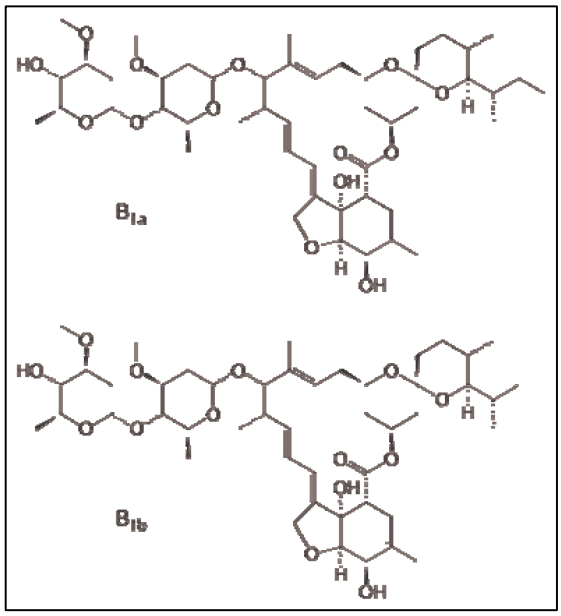

Figure 10: Structure of Ivermectin.
(Source: wikipedia.org)

Albendazole

Albendazole (Figure 11) is a member of the benzimidazole family of compounds. It functions by binding to the colchicine-sensitive site of β-tubulin subunits. Microtubules are composed of α- and β-tubulin subunits and are involved in a large variety of intracellular functions such as transport of vesicles within the cell. They form by polymerization of the subunits at the positive end, at the same time as depolymerization at the negative end (Stryer, 1995b). The polymerization event can be inhibited by capping the positive end either with colchicine or benzimidazole. It has been shown in cestodes and nematodes that inhibition was localized to the intestinal cells and tegument, leading to a loss in the transport of secretory vesicles. This resulted in a decreased glucose uptake, decrease in energy production and depletion of the glycogen reserve. These events lead to immobilization and gradual death of worms (Martin *et al.*, 1997).

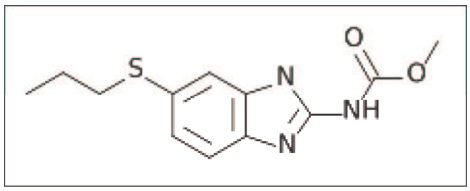

Figure 11: Structure of Albendazole.
(Source: wikipedia.org)

Diethylcarbamazine

Diethylcarbamazine (Figure 12) has been used to treat LF for more than 30 years yet its mode of action is not well understood. It has been shown to have an inhibitory effect on arachidonic acid metabolism of the worm and host by interfering with the production of protacyclin and prostaglandin E. This leads to a tightening of the blood vessels, hence provoking accumulation of granulocytes and platelets, and activation of the innate immune response (Martin *et al.*, 1997).

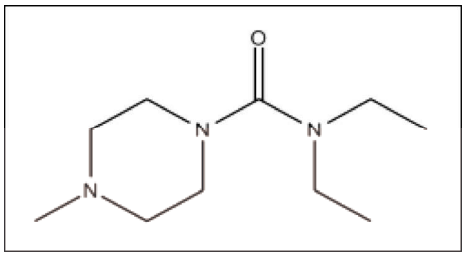

Figure 12: Structure of diethylcarbamazine.
(Source: wikipedia.org)

All 3 drugs described target the immature stages of filarial parasites (microfilariae and larval stages) and lengthy treatments are required to cover the reproductive lifespan of the long-lived adult worms (up to 15 years for *O. volvulus*) in order to interrupt transmission. The main limitations of these drugs are their limited effects on adult worms and emergence of drug resistance. Resistance has been evident for some time in animals treated for intestinal nematode infection following widespread usage of the same drug classes (Waghorn, 2006). IVM resistance in humans has been recently documented in humans infected with *O. volvulus* (Prichard 2007). Therefore there is an urgent need to identify new drug targets and develop new therapies for human filarial infections.

Wolbachia as a drug target

In recent years, obligate α-proteobacterial endosymbionts of the genus *Wolbachia* that are present in most filarial nematodes (Kozek and Marroquin, 1977) have been investigated as a new approach to chemotherapy. They are found in both male and female worms, as well as in all larval stages. Their presence in oocytes, developing eggs and microfilaria (McLaren *et al.*, 1975; Kozek and Marroquin, 1977) indicates that the bacteria are maintained in the population by vertical transmission. They are predominantly localized in the lateral chords and the oocytes

(Figure 13) (Hoerauf and Pfarr, 2007). These bacteria are essential for worm development, fertility and survival (Hoerauf *et al.*, 2000; Taylor *et al.*, 2005; Hoerauf and Pfarr, 2007; Pfarr and Hoerauf, 2007)). Antibiotics (mainly of the tetracycline family) have been shown experimentally to be effective against *Wolbachia* and depletion of bacteria results in death of filarial worms (Taylor *et al.*, 2005; Hoerauf, 2006). Unfortunately, the high dosage and lengthy regimen required to have this effect precludes the use of antibiotics for control of filarial infections at the moment. Nevertheless, these studies demonstrate the feasibility of developing treatments targeting the *Wolbachia* endosymbiont (Hoerauf *et al.*, 2000; Pfarr and Hoerauf, 2006).

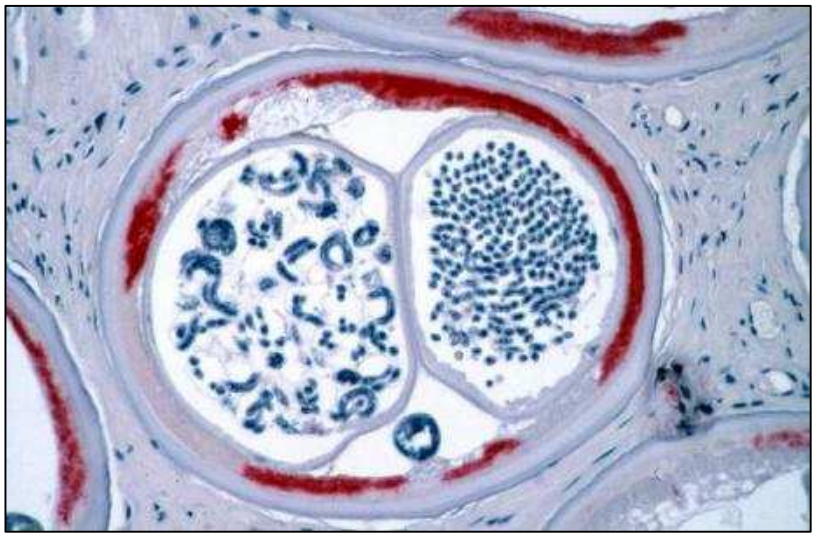

Figure 13: *Wolbachia* stained in red in the hyopdermal tissue of a section of the filarial nematode host (*O. volvulus*).
(Source: authors Mark Blaxter, Achim Hoerauf, Dietrich Buttner. www.eol.org)

Drug discovery process

The drugs in use today for the treatment of human filariasis were discovered in the veterinary industry. The discovery process largely involved screening of chemical compound and small molecule collections for activity against live parasites either in culture or in infected animals. This approach requires large amounts of parasite material and large compound librairies. The recent availability of genomic information and automation of in high throughput screening methods have allowed researchers to refine their way of looking for new drugs (Woods *et al.*, 2007; Kaminsky *et al.*, 2008). Various options are now available as described previously (Hudson and Nwaka, 2007). These include: *biologically-driven compound selection* where 'bioactive compounds' from companies with veterinary/agrochemical anthelmintic and insectide

programs are evaluated, this screening may be performed using live *Caenorhabditis elegans* (free-living model nematode) which is amenable to high throughput screening (HTS); and *biochemically-driven compound selection* where families of compounds from non-parasite pharma programs are tested in whole worm screens, or HTS are performed against specific parasite targets.

Using genomic and functional (C. elegans) data to identify new drug targets

The recently completed genome sequences of *B. malayi* (Ghedin *et al.*, 2007) and its *Wolbachia* endosymbiont (*w*Bm) (Foster *et al.*, 2005a) have provided a tremendous opportunity for the discovery of new drug targets. However, validation of drug targets represents a challenge since functional genomic technologies are limited (such as RNA interference (RNAi) in filarial worms) or non-existent (for filarial *Wolbachia*) in these systems. To circumvent this problem our laboratory has used the related free-living nematode *C. elegans* as a surrogate for *B. malayi*. *C. elegans* has been proposed on several occasions as a good model for other nematodes (Aboobaker and Blaxter, 2000; Britton and Murray, 2006; Kaletta and Hengartner, 2006; Holden-Dye and Walker, 2007). *C. elegans* belongs to clade V in the Order Rhabditida (Figure 3). It is genetically tractable, easy to maintain on petri dishes in the laboratory (feeding on an *E. coli* lawn) and has a simple life cycle (Figure 14). Embryogenesis occurs in about 12 hours, development through 4 larval stages to the adult stage occurs in 2.5 days, and the life span is 2-3 weeks. A great deal is known about the development of this organism because it is small (1 mm in length) and transparent. It has been used extensively as a research model for developmental biology, genetics, neurobiology and cell biology for more than 35 years.

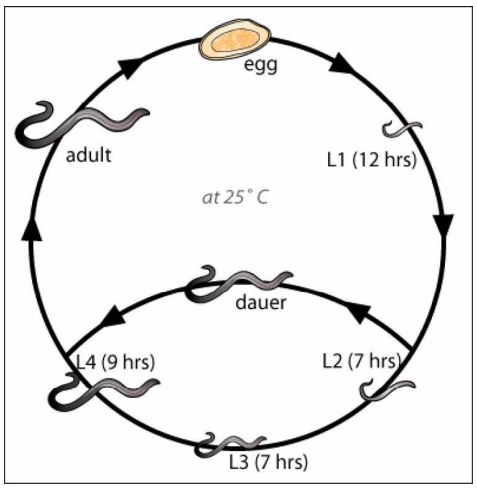

Figure 14: *C. elegans* life cycle.
(source: www.scq.ubc.ca)

C. elegans has a fully sequenced and extensively annotated genome. Several genome-wide RNAi studies have been performed and this data is publicly available in Wormbase (www.wormbase.org) (Bieri *et al.*, 2007). Our lab has used this information to develop a genome-wide rational drug target selection methodology to identify potential new drug targets. Firstly, the potentially essential genes in *B. malayi* are predicted by performing sequence comparisons between the 2 genomes and identification of orthologs of *C. elegans* genes that have any RNAi phenotype. The assumption is that these orthologs will perform the same function in the filarial parasite. Secondly, genes that have high homology to mammalian genes are removed. The remaining genes are ranked further using a custom algorithm to generate a pool of 'validated drug targets' (Figure 15) (Foster *et al.*, 2005b; Kumar *et al.*, 2007). The algorithm was designed to predict the efficacy and potential of each candidate target. Some of the parameters taken into account were the severity of the phenotype observed in *C. elegans*, the level of identity with the model organism gene, as well as when a certain gene was expressed (if the data are available). Predicted druggability and expressability were also taken into account. A similar approach has been used in another lab using fragmentary EST sequence data (McCarter, 2004).

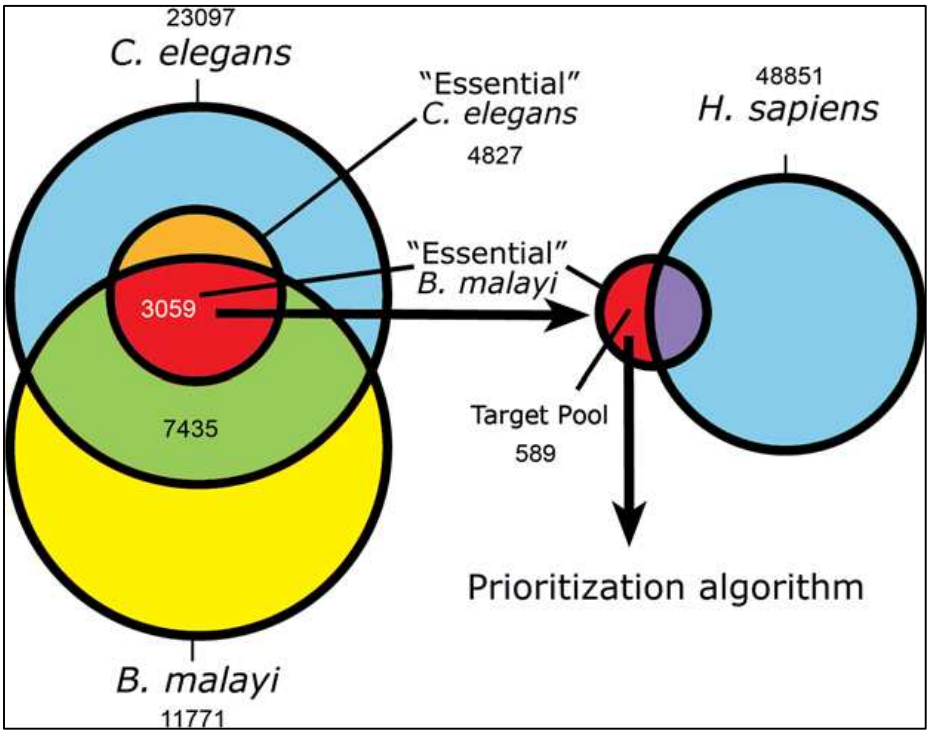

Figure 15: Genomic filtering method to identify potential drug targets in *B. malayi*.
The number of genes in each category are indicated.
(Source: (Kumar *et al.*, 2007))

Cofactor independent phosphoglycerate mutase as a potential drug target in filarial nematodes and Wolbachia

One particularly interesting target identified using this approach is the enzyme co-factor independent phosphoglycerate mutase (iPGM). PGMs catalyze the interconversion of 2- and 3-phosphoglycerate (2-PG and 3-PG) in the glycolytic and gluconeogenic pathways. Although these pathways are highly conserved among different organisms, two distinct PGM enzymes are known to exist, iPGM and the cofactor dependent phosphoglycerate mutase, dPGM. Mammals, in addition to some bacteria and fungi, possess exclusively dPGM whereas other organisms may have exclusively iPGM, or both forms (Carreras *et al.*, 1982; Fothergill-Gilmore and Watson, 1990; Fraser *et al.*, 1999). The two form have different length (iPGM, 420 amino acids (aa); dPGM, 210 aa) and they share no sequence homology and operate via different biochemical mechanisms (Jedrzejas *et al.*, 2000b; Bond *et al.*, 2001).

iPGM belongs to the alkaline phosphatase superfamily. It catalyzes the transfer of phosphate through a serine residue and is insensitive to vanadate (Britton *et al.*, 1971; Jedrzejas and Setlow, 2001; Jedrzejas, 2002; Rigden *et al.*, 2003). It functions as a monomer and contains two domains, one bearing structural similarity to alkaline phosphatase, and the other to sulfatase (Jedrzejas, 2000). Detailed biochemical and structural studies have been performed on the bacterial iPGM enzymes from *Bacillus stearothermophilus* and *Bacillus subtilis* (Jedrzejas *et al.*, 2000a; Nukui *et al.*, 2007). iPGM has also been identified in a number of pathogens including the protozoan parasites *Trypanosoma brucei* (Chevalier *et al.*, 2000; Collet *et al.*, 2001) and *Leishmania mexicana* (Guerra *et al.*, 2004) where it is being pursued as a drug target.

dPGM belongs to the acid phosphatase superfamily, bearing some amino acid similarity to acid phosphatase and fructose 2,6 bisphosphatase (Jedrzejas, 2000). Mammals possess only this form of enzyme. It requires the cofactor 2,3 bis phosphoglycerate to transfer phosphate between the substrate and the product through a phosphohistidine intermediate (Rigden *et al.*, 2002; Rigden *et al.*, 2003), and is sensitive to vanadate (Carreras *et al.*, 1980). It can function as a monomer, dimer (in *E. coli* and humans) or tetramer (in yeast). The structures of several dPGMs have been determined, including the enzymes from *E. coli* (Bond *et al.*, 2001), *Mycobacterium tuberculosis* (Muller *et al.*, 2005) and yeast (Rigden *et al.*, 1998) to cite a few.

Previous studies in our lab have shown that nematodes possess only the iPGM form and

that it has an essential role in *C. elegans* development, indicating that it is potential drug target in parasitic nematodes (Figure 16) (Zhang et al., 2004). Our interest in developing iPGM further as a drug target led us to a collaborative study with Dr. Najib El-Sayed (George Washington University, formerly of TIGR) on the parasite *Trypanosoma brucei*, the cause of sleeping sickness or African trypanosomiasis in humans.

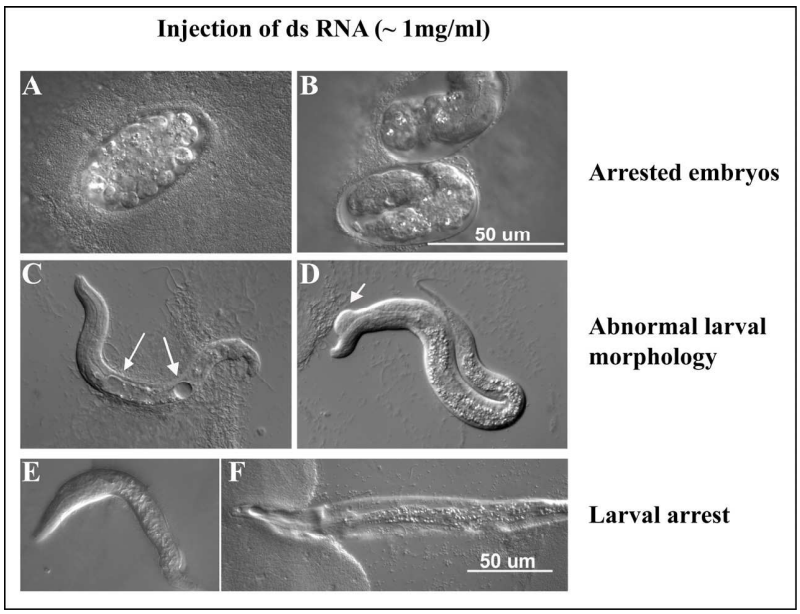

Figure 16: Differential interference contrast images of abnormal embryos and larvae resulting from RNAi knockdown of *Ce-iPGM*.

Embryos that failed to hatch were arrested at various stages during embryogenesis including early (A) and late stages (B). Variable phenotypes were observed in larvae including degenerating intestinal cells that contain large vacuoles (C, arrows) and variable abnormal body morphologies (D, arrow). Some larvae arrested at L1 (E) or died (F) (adapted from (Zhang *et al.*, 2004)).

Cofactor independent phosphoglycerate mutase as a drug target in African trypanosomes

Human African trypanosomiasis was first identified in 1721 by John Atkins (Cox, 2002). Two parasites of the Trypanosomatidae family are responsible for the disease. The chronic form is caused by *Trypanosoma brucei gambiense* in West Africa, whereas *T. b. rhodensiense*, causes a more acute and severe disease (Figure 17) in East and South Africa. Both are transmitted by tsetse flies (*Glossina* spp).

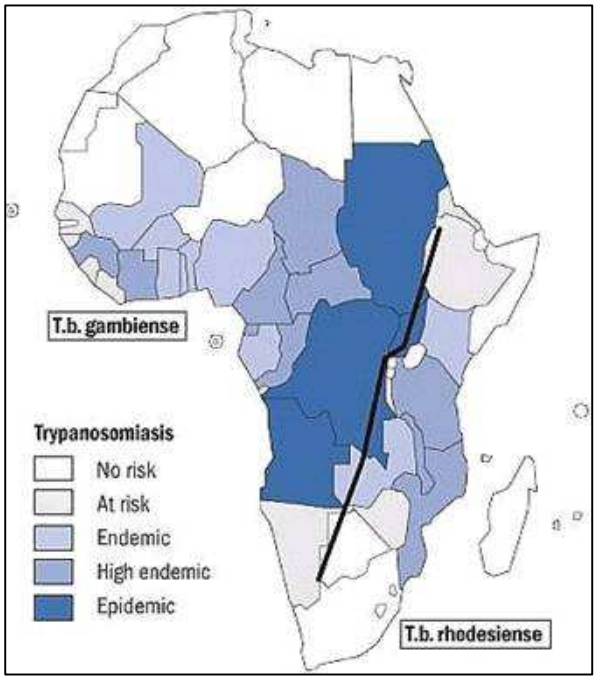

Figure 17: Distribution of *T. b gambiense* and *T. b. rhodesiense* in Africa.
(Source: www.medicalecology.org)

6,000 to 10,000 human cases are documented annually, and 35 million people are at risk (WHO). Commonly, after infection, the patient has a skin reaction called a trypanosomal chancre (Figure 18). The parasite invades the central nervous system, resulting in chronic encephalopathy with general degradation of cognitive functions until the patient enters a terminal somnolent stage (Barrett *et al.*, 2003; Croft *et al.*, 2005).

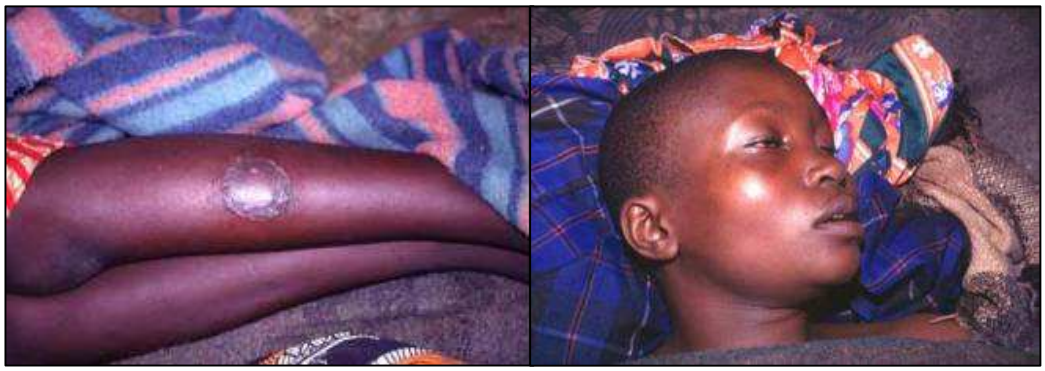

Figure 18: Symptoms of African sleeping sickness.

Left panel: Presentation of a trypanosomal chancre at the biting site. Right panel: Young girl at the last stage of infection. (Source: www.standford.edu)

T. brucei are protist unicellular parasites of the Kinetoplastid class. The are 4 distinct forms of the parasite (Figure 19): bloodstream (in mammalian hosts), procyclic trypomastigotes (in the midgut of the tsetse fly vector), epimastigotes, metacyclic trypomastigotes (in the salivary gland of the tsetse fly) (Dreesen *et al.*, 2007).

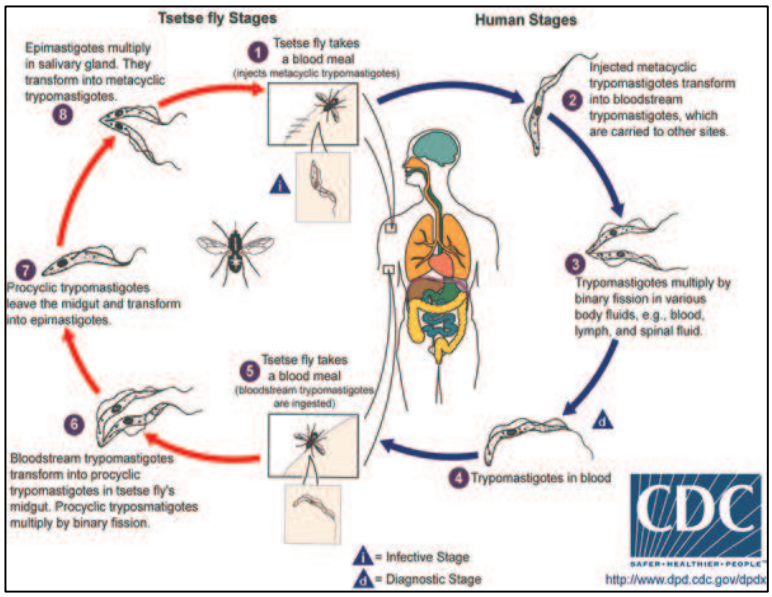

Figure 19: Life cycle of *T. brucei.*

(Source: http://www.dpd.cdc.gov/dpdx)

Diagnosis of sleeping sickness is normally achieved by identifying the parasites in the blood, cerebrospinal fluid or lymph node aspirate. ELISA and agglutination tests can also be used to detect an antigen (CATT) or antibodies (CIATT, indirect agglutination test) (Muller and Baker, 1990).

There are a number of drugs used for the treatment of sleeping sickness, however, there are issues with toxicity and inefficacy during the late stage of the disease, and resistance is also appearing for most of them (Croft *et al.*, 2005; Delespaux and de Koning, 2007). Suramin and pentamidine are used in the early stage of the disease. Suramin is a naphthalene derivative first used against sleeping sickness in 1922. Its mode of action is linked to the fact that as a large polyanion molecule, it binds to a vast variety of proteins (for example dihydrofolate reductase, reverse transcriptase, receptor mediated uptake of low density lipoprotein, thymidine kinase). It is still unclear which event(s) confer the trypanocide activity but it is believed that the blocking of LDL contributes to the mode of action (Barrett and Barrett, 2000; Delespaux and de Koning, 2007). Pentamidine is believed to attach to DNA thus inhibiting DNA replication. The exact mechanism is still unclear but it is known that there is an active uptake of the molecule through 3 types of transporter: the P2 amino purine transporter, and the high and low affinity pentamidine transporters (HAPT, LAPT) (Delespaux and de Koning, 2007).

Three other drugs are available for the treatment of patients with late stage disease. Melarsoprol which is a toxic organic compound of arsenic, requires treatment under constant surveillance of a doctor. The drug has considerable side effects but it is still the only drug effective against both *T. b. gambiense* and *T. b. rhodensiense*, in the late stage of the disease. Its mechanism of action is not totally understood but its accumulation happens through a P2-transporter (Croft *et al.*, 2005; Delespaux and de Koning, 2007). Eflornithine (α-difluoromethylornithine or DFMO) is a far less toxic derivative and is only active against *T. b. gambiense*. It is a suicide inhibitor of the enzyme ornithine decarboxylase, which regulates cell division by catalyzing the first step in polyamine biosynthesis, and therefore inhibits the formation of putrescine. Another compound Nifurtimox is used in association with melarsoprol. Nifurtimox results in production of superoxide anions and H_2O_2 which are thought to be toxic to the parasite (Maya *et al.*, 1997). This compound is administered orally, and appears to be efficient in melarsoprol resistant patients (Delespaux and de Koning, 2007).

Genomic analysis of the glycolysis pathways in nematodes and Wolbachia

The discovery of iPGM as a potential drug target in nematodes and *w*Bm prompted us to perform further genome analysis of the glycolytic/gluconeogenic pathway in these organisms. Glycolysis involves the transformation of glucose into pyruvate and normally occurs via the Embden-Meyerhof pathway. In some bacteria, an alternative pathway exists, namely the Entner-Doudoroff Pathway (Conway, 1992). Glycolysis occurs in a wide variety of cells, and in most organisms, and it is considered one of the most ancient metabolic pathways (Romano and Conway, 1996). In most eukaryotes and prokaryotes, glycolysis takes place in the cytosol. However, the situation may differ in protozoa and plants as some of the glycolytic enzymes are found in chloroplasts (plants) and glycosomes (*T. brucei*).

Classical glycolysis (Figure 20) involves 10 steps, where steps 1, 3 and 10 are irreversible when performed by the standard enzymes (Stryer, 1995a). In classical gluconeogenesis, where glucose is produced from pyruvate, other enzymes are involved in order to reverse the pathway. The pyruvate is converted to phosphoenolpyruvate using pyruvate and phosphoenolpyruvate carboxylases (EC 6.4.1.1 and EC 4.1.1.31 respectively, reverse reaction 10). Fructose 1,6 bisphosphate is converted to fructose 6 phosphate using fructose 1,6 bisphosphatase (EC 3.1.3.11, reverse reaction 3). Glucose 6 phosphate is converted to glucose using glucose 6 phosphatase (EC 3.1.3.9, reverse reaction 1) (Stryer, 1995a). Glycolysis results in most cases in the net gain of two ATP molecules and the production of NADH, thereby providing energy to the cell. The pyruvate formed can then enter into the TCA cycle or undergo fermentation. Five of the standard glycolytic enzymes have alternatives (Figure 20) and the distribution of those enzymes in the organisms included in my Ph.D studies is shown (Table 1). The alternative enzymes for steps 3 and 10 differ from the standard enzymes in that they are reversible. The mammalian and *E. coli* enzymes are included for comparison.

Our analysis revealed that the *Wolbachia* endosymbiont lacks pyruvate kinase (PK) and may instead utilize the enzyme pyruvate phosphate dikinase (PPDK; ATP:pyruvate, orthophosphate phosphotransferase, EC 2.7.9.1), which can potentially fulfill both the glycolytic and gluconeogenic conversion. Interestingly, it seems that glycolysis in *Wolbachia* start with the fructose 1,6 bisphosphate, while the presence of fructose 1,6 bisphosphatase would allow gluconeogenesis to proceed up to fructose 6 phosphate (Foster *et al.*, 2005a).

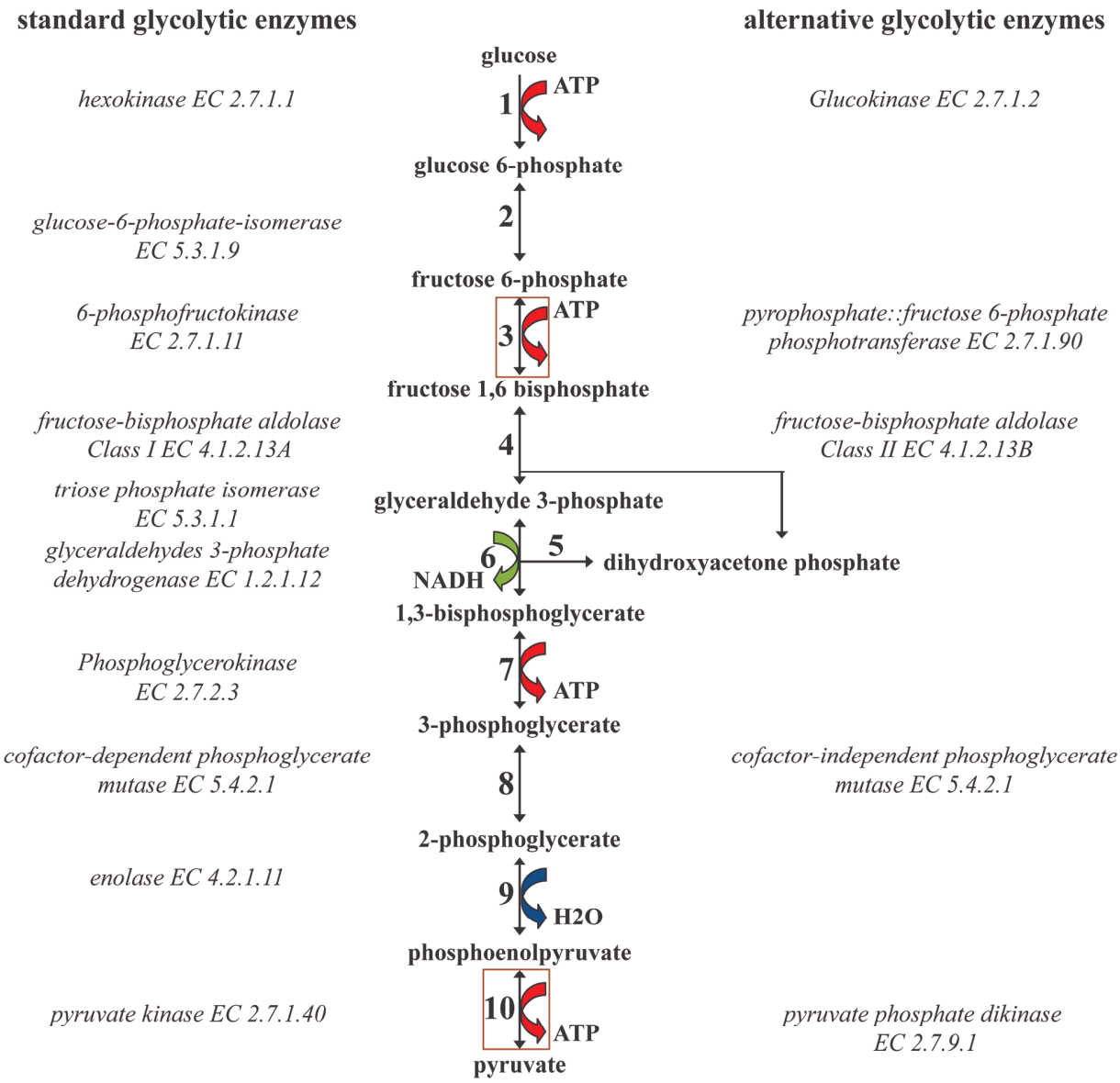

Figure 20: Glycolysis pathway using standard and alternative enzymes.

The boxes indicate those reactions (3 and 10) which are reversible when performed by the alternative enzymes.

Enzymes Standard/ alternative	*Mammals*	*E. coli*	*T. brucei*	*C. elegans*	*B. malayi*	*O. volvulus*	*w*Bm
HEX / GLK	Both	GLK	HEX	HEX	HEX	unknown	Absent
GPI	present	present	present	present	Present	Present	Absent
PFK / PFP	PFK	PFK	PFK	PFK	PFK	PFK	Absent
FBA I / FBA II	FBA I	FBA I	FBA I	FBA I	Unknown	FBA I	FBA I
TPI	present	present	present	present	Present	present	present
GAP	present	present	present	present	Present	present	present
PGK	present	present	present	present	Present	present	present
dPGM / iPGM	dPGM	Both	iPGM	iPGM	iPGM	iPGM	iPGM
ENO	present	present	present	present	Present	present	present
PK / PPDK	PK	PPDK	Both	PK	PK	PK	PPDK

Table 1: Distribution of the standard and alternative glycolytic enzymes in the organisms studied.
The enzymes presented in the table are HEK/GLK (hexokinase/glucokinase), GPI (glucose-6-phosphate-isomerase), PFK/PFP (6-phosphofructokinase/pyrophosphate::fructose 6-phosphate phosphotransferase), FBA I/FAB II (fructose-bisphosphate aldolase Class I/class II), TPI (triose phosphate isomerase), GAP (glyceraldehyde 3-phosphate dehydrogenase), PGK (Phosphoglycerokinase) dPGM/iPGM (cofactor-dependent/ cofactor-independent phosphoglycerate mutase), ENO (enolase) and PK/PPDK (pyruvate kinase/pyruvate phosphate dikinase).

Pyruvate phosphate dikinase as a drug target in Wolbachia

PPDK is a ~96 kDa protein which functions as a dimer (e.g *Trypanosoma brucei* (Cosenza *et al.*, 2002)) or a tetramer (e.g. *Entamoeba histolytica* (Saavedra-Lira *et al.*, 1998)), and catalyses the reversible conversion of AMP, PPi and phosphoenolpyruvate into ATP, Pi and pyruvate (Wood *et al.*, 1977)**.** The reaction catalysed by PPDK occurs in three steps (Xu *et al.*, 1995), where the outcome depends on the organism (glycolysis and ATP formation, or phosphoenolpyruvate synthesis):

E-His + ATP + Pi ↔ E-His-PP·AMP·Pi

E-His-PP·AMP·Pi ↔ E-His-P + AMP + PPi

E-His-P + Pyruvate ↔ E-His + PEP

PPDK is an energy conserving, reversible alternative to PK producing four ATP molecules per glucose molecule compared with the two produced by PK in standard glycolysis

(Mertens, 1993). The enzyme is composed of three domains each with an important role. The central domain is small and spherical and its catalytic histidine residue is responsible for the transport of the phosphate group between the active sites of the two terminal domains, while the N-terminal domain (containing the ATP/Pi active site) catalyzes the first two steps of the reaction, and the C-terminal domain (containing the pyruvate active site) performs the third step of the reaction (Ye *et al.*, 2001; Herzberg *et al.*, 2002). It has been speculated that the enzyme might have evolved through the process of gene duplication and fusion leading to the linkage of three proteins whose structures coevolved to support a novel function (Lin *et al.*, 2006).

Most organisms, including mammals possess exclusively PK. Therefore the absence of PPDK in mammals makes the enzyme an attractive *Wolbachia* drug target. Since there is no protein sequence homology between PPDK and PK, it is feasible that highly specific inhibitors of PPDK activity may be identified. PPDK has also been found in protozoan parasites and its important role in *Entamoeba histolytica* and *Giardia lamblia* (Saavedra-Lira and Perez-Montfort, 1996; Feng *et al.*, 2008) has stimulated considerable interest in the enzyme as a potential target for the design of novel anti-protozoan drugs. PPDK is considered particularly important in trypanosomes since it appears to provide a link between breakdown of sugars and fatty acids via glycolysis and fatty acid β-oxidation for other biosynthetic pathways (Acosta *et al.*, 2004). *T. brucei* PPDK is the most characterized of the protozoan enzymes (Bringaud *et al.*, 1998) and a 3.0 Å resolution crystal structure is available (Cosenza *et al.*, 2002) which shows considerable similarity to the structure of the bacterial (*Clostridium symbiosum*) enzyme (Herzberg *et al.*, 1996).

Goal of the present study

One of the goals of my Ph.D studies was to study nematode iPGM enzymes in more detail with a view to determining if the filarial and *C. elegans* iPGM enzymes possess similar biochemical characteristics which might indicate that a single enzyme inhibitor would likely be effective against all nematode enzymes (Chapter 1). This information would also indicate the validity of using live *C. elegans* worms in a screen to evaluate the efficacy of enzyme inhibitors. Interestingly, our genome analysis indicated that the enzyme may also be present in the *Wolbachia* endosymbiont. Therefore we were interested in cloning and expressing the putative

iPGM to determine if this might also be a potential drug target (Chapter 2). Our interest in developing iPGM further as a drug target led us to a collaborative study with Dr. Najib El-Sayed (George Washington University, formerly of TIGR) on the parasite *Trypanosoma brucei*, the cause of sleeping sickness or African trypanosomiasis in humans (Chapter 3).

From the genomic analysis of the glycolysis of *Wolbachia* we hypothesized that PPDK could be a good drug target, we were therefore interested in cloning and expressing the predicted PPDK from *Wolbachia* (*w*Bm-PPDK) to gain insight into the energy metabolism of this endosymbiont and to develop the enzyme as a drug target (Chapter 4).

The ultimate goal of our studies on iPGM and PPDK is the discovery of specific inhibitors that may represent lead compounds for further development as anti-parasitic compounds. During my studies, I screened several phage display libraries for inhibitors of iPGM (Chapter 5). I also performed biochemical studies on recombinant *E. coli* iPGM and dPGM to parallel a study developing mutant strains that were constructed for use in cell-based screens for specific inhibitors of iPGM activity, and for studies on the roles of the two enzymes in *E. coli* (Chapter 6).

Results

Chapter 1:
Molecular and biochemical characterization of nematodes independent phosphoglycerate mutase

Caractérisation moléculaire et biochimique des phosphoglycérates mutases indépendantes de nématodes

Dans cet article, nous décrivons le clonage et l'expression de la phosphoglycérate mutase cofacteur indépendante d'*Onchocerca volvulus*, la filaire humaine responsable de la cécité des rivières. Notre but a été de comparer l'enzyme d'*O. volvulus* avec la PGMi de la filaire lymphatique *B. malayi* et la PGMi plus distante du nématode *C. elegans*. Malheureusement, la séquence du génome d'*O. volvulus* n'est pas disponible. Cependant, j'ai été en mesure d'identifier un certain nombre d'ESTs correspondantes à la PGMi dans la base de données du NCBI. J'ai utilisé ces informations pour concevoir des amorces afin d'amplifier le gène. J'ai ensuite effectué une analyse de séquence pour l'enzyme d'*O. volvulus*. Afin de produire l'enzyme recombinante, j'ai utilisé le système d'expression de protéine His-tag, qui avait déjà été utilisé avec succès dans le laboratoire pour produire les PGMis actives de *B. malayi* et *C. elegans*. Pour les études biochimiques détaillées, j'ai fraîchement produit les PGMis de *O. volvulus*, *B. malayi* et *C. elegans*. J'ai également inclus la PGMd humaine dans certaines des études pour mettre en évidence les différences entre les deux formes de PGMs. L'essai standard pour la mesure de l'activité implique l'utilisation de 3 enzymes de couplages, ce qui peut rendre les analyses compliquées. J'ai par conséquent conçu un essai en deux étapes qui m'a permis de déterminer les optimums de pH et de température pour les PGMis, et cela indépendamment des effets de ces mêmes paramètres sur les enzymes de couplage. Le problème technique le plus difficile à résoudre a été de déterminer la préférence ionique des PGMs de nématodes. Nous avons découvert que ces enzymes étaient particulièrement sensibles à l'effet de l'EDTA et difficiles à réactiver. Après avoir exploré un certains nombre de protocoles, nous avons découvert qu'une inactivation partielle, utilisant un protocole publié dans une étude de la PGMi de *T. brucei*, nous a permis de résoudre nos problèmes et d'évaluer des ions variés. Les conditions optimales ont ensuite été utilisées pour mesurer les différents paramètres cinétiques.

Les résultats de cette étude démontrent que les PGMis de filaires et de *C. elegans* possèdent des caractéristiques biochimiques similaires et indiquent qu'un unique inhibiteur serait sûrement efficace contre toutes les enzymes de nématodes. Nos données

supportent l'utilisation de *C. elegans* en tant qu'outil de criblage primaire pour tester des inhibiteurs enzymatiques à activité anti-nématodes.

Molecular and biochemical characterization of nematode cofactor independent phosphoglycerate mutases

In this paper we describe the cloning and expression of cofactor-independent phosphoglycerate mutase from *Onchocerca volvulus,* the human filarial parasite responsible for river blindness. Our goal was to compare the *O. volvulus* enzyme with the iPGM from the lymphatic filarial parasite *B. malayi* and the more distantly related iPGM from the free-living nematode *C. elegans*. Unfortunately, the genome sequence for *O. volvulus* is unavailable, however I was able to identify a number of *O. volvulus* ESTs corresponding to iPGM in the NCBI databases. I used this information to design specific primers to successfully amplify the gene. I then performed sequence analysis of the *O. volvulus* enzyme. In order to produce recombinant enzyme I used the His-Tag protein expression system, which had been used in the lab previously to produce active *B. malayi* and *C. elegans* iPGM enzymes. For the detailed biochemical studies, I prepared fresh iPGM enzyme from *O. volvulus*, *B. malayi*, and *C. elegans*. I also included human dPGM in some of my experiments to highlight the differences between the two forms of PGM enzyme. The standard assay to measure PGM activity is a coupled assay involving 3 coupling enzymes, which can complicate the analyses. I therefore established a two-step assay in the lab that allowed me to determine pH and temperature optima for the PGMs independently of the effect of those parameters on the coupling enzymes. The most technically challenging aspect of this study was determining the ion requirement and preference of the nematode PGM enzymes. We discovered that the enzymes were particularly sensitive to EDTA and difficult to re-activate. After exploring a number of different protocols we found that partial in-activation, using a procedure published in a *T. brucei* iPGM study enabled us to move forward and evaluate various ions. The optimal conditions for enzyme activity were then used to calculate various kinetic parameters.

The results of this study demonstrate that the filarial and *C. elegans* iPGM enzymes possess similar biochemical characteristics and indicate that a single enzyme inhibitor would likely be effective against all nematode enzymes. Our data supports the

use of *C. elegans* as a primary screen for testing enzyme inhibitors for anti-nematode activity.

Chapter 1

Molecular and biochemical characterization of nematode cofactor independent phosphoglycerate mutases

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***Abbreviations*:** iPGM, cofactor-independent phosphoglycerate mutase; dPGM, cofactor-dependent phosphoglycerate mutase; 2-PG and 3-PG, 2- and 3-phosphoglycerate; *Ce*, *Caenorhabditis elegans*; *Bm*, *Brugia malayi*; *Ov*, *Onchocerca volvulus*; *Hs*, *Homo sapiens*.

Note: The nucleotide sequence reported in this paper is available in GenBank™ Data Bank under the accession number **AY640434**.

Résumé

Les phosphoglycérates mutases (PGM, EC 5.4.2.1) catalysent l'isomérisation du 3-phosphoglycérate et du 2-phosphoglycérate dans la glycolyse et la néoglucogenèse. Deux types de PGMs existent dans la nature, un type qui requiert le 2,3-bisphosphoglycérate en tant que cofacteur (PGMd) et un autre type qui ne le requiert pas (PGMi). Les deux enzymes sont structurellement distinctes et possèdent des mécanismes réactionnels différents. Dans un organisme donné, l'une, l'autre ou les deux formes peuvent exister. Les nématodes possèdent la forme indépendante tandis que les mammifères ont la phosphoglycérate mutase dépendante. Dans cette étude, nous avons cloné et exprimé la PGMi d'*Onchocerca volvulus*. Nous décrivons ensuite les propriétés catalytiques des PGMis d'*O. volvulus*, *Brugia malayi* et *Caenorhabditis elegans*. Les températures et pH optimaux ont été déterminés pour chaque enzyme. Tout comme les autres PGMis, l'activité des enzymes de nématodes est dépendante de la présence d'ions divalents. Une inactivation par l'EDTA a pu être inversée le plus efficacement par les ions magnésium et manganèse. Les paramètres cinétiques et activités spécifiques des différentes enzymes recombinantes ont été déterminés. La grande similitude des propriétés catalytiques des trois enzymes indique qu'un seul inhibiteur enzymatique serait sûrement efficace contre toutes les enzymes de nématode. L'inhibition de la PGMi *in vivo* peut induire la létalité comme l'ont indiqué les études par ARN interférence chez *C. elegans*. Nos résultats soutiennent le développement de la PGMi en tant que cible thérapeutique prometteuse chez les nématodes parasitaires.

***Mot-clés* :** *Onchocerca volvulus*, *Brugia malayi*, *Caenorhabditis elegans*, Glycolyse, Néoglucogenèse, Phosphoglycérate mutase

Abstract

Phosphoglycerate mutase (PGM, EC 5.4.2.1) catalyzes the isomerization of 3-phosphoglycerate and 2-phosphoglycerate in glycolysis and gluconeogenesis. Two distinct types of PGM exist in nature, one that requires 2,3-bisphosphoglycerate as a cofactor (dPGM) and another that does not (iPGM). The two enzymes are structurally distinct and possess different mechanisms of action. In any particular organism, one form may exist or both. Nematodes possess the iPGM form whereas mammals have dPGM. In the present study, we have cloned and expressed iPGM from *Onchocerca volvulus* and described the catalytic properties of *O. volvulus*, *Brugia malayi* and *Caenorhabditis elegans* iPGM enzymes. Temperature and pH optima were determined for each enzyme. Like other iPGM enzymes, the activities of the nematode iPGM enzymes were dependent on the presence of divalent ions. Inactivation by EDTA could be restored most effectively by magnesium and manganese ions. Kinetic parameters and specific activities of the various recombinant enzymes were determined. The high similarity in catalytic properties among the enzymes indicates that a single enzyme inhibitor would likely be effective against all nematode enzymes. Inhibition of iPGM activity *in vivo* may lead to lethality as indicated by RNAi studies in *C. elegans*. Our results support the development of iPGM as a promising drug target in parasitic nematodes.

Keywords: *Onchocerca volvulus*, *Brugia malayi*, *Caenorhabditis elegans*, Glycolysis, Gluconeogenesis, Phosphoglycerate mutase

Molecular and biochemical characterization of nematode cofactor independent phosphoglycerate mutases

Sylvine RAVERDY, Yinhua ZHANG, Jeremy FOSTER and Clotilde K.S. CARLOW

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Chapter 2: Expression of phosphoglycerate mutase from the *Wolbachia* endosymbiont of *Brugia malayi* in the yeast *Kluyveromyces lactis*

Expression de la phosphoglycérate mutase de *Wolbachia* symbiote de *Brugia malayi* dans la levure *Kluyveromyces lactis*

Dans cette publication, nous décrivons le clonage et l'expression de la phosphoglycérate mutase indépendante (PGMi) de *Wolbachia*, symbiote intracellulaire de *Brugia malayi* (*w*Bm). Nous avons par le passé montré que la PGMi est une cible thérapeutique valide chez les nématodes et nous avons découvert que le génome *w*Bm codait pour une putative PGMi. Notre but a été de cloner et exprimer cette putative *w*Bm-PGMi afin de vérifier son activité et son possible rôle dans le métabolisme du symbiote, ainsi que de la considérer en tant que cible thérapeutique. Malheureusement, les protocoles utilisés pour produire les PGMis active de nématodes et autres organismes ne fonctionnèrent pas pour *w*Bm-PGMi. Un certain nombre de systèmes d'expression bactérienne furent utilisés, et divers paramètres ont été explorés pour optimiser l'expression et la purification de la protéine. J'ai participé à ces études et effectué les essais d'activité sur toutes les protéines recombinantes. Dans certains cas j'ai modifié le test enzymatique afin de permettre la mesure d'activité avec de très petites quantités de protéine purifiée. Finalement nous avons été capable de produire la *w*Bm-PGMi active dans la levure *Kluyveromyces lactis* en utilisant une nouvelle méthode qui permet l'expression cytoplasmique de protéines recombinantes. Ce protocole peut être d'intérêt général pour ceux travaillant avec des protéines difficiles à exprimer. Nos résultats supportent le développement de *w*Bm-PGMi en tant que cible thérapeutique potentielle chez les filaires.

Expression of phosphoglycerate mutase from the *Wolbachia* endosymbiont of *Brugia malayi* in the yeast *Kluyveromyces lactis*

In this paper, we describe the cloning and expression of the independent phosphoglycerate mutase (iPGM) from the *Wolbachia* endosymbiont of *Brugia malayi (w*Bm). We had previously shown that iPGM is a valid drug target in nematodes and discovered that the *w*Bm genome encodes a putative iPGM. Our goal was to clone and express the putative *w*Bm-iPGM protein in order to verify its activity and possible role in the energy metabolism of the endosymbiont, as well as consider it further as a drug target. Unfortunately, the protocols that were used to produce active recombinant iPGM from nematodes and other organisms were unsuccessful for *w*Bm-iPGM. A number of different bacterial expression systems were explored, and various parameters were investigated to optimize expression and purification of the protein. I participated in these studies and performed the activity assays on all recombinant proteins. In some cases, I modified the assay to enable testing of very small amounts of purified protein. Finally we were able to produce active *w*Bm-iPGM in the yeast *Kluyveromyces lactis* using a new procedure that results in cytoplasmic expression of recombinant protein. This protocol may of general interest to those working with difficult to express proteins. Our results support the development of *w*Bm-iPGM as a potential drug target in filarial nematodes.

Expression of phosphoglycerate mutase from the *Wolbachia* endosymbiont of *Brugia malayi* in the yeast *Kluyveromyces lactis*

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Abbreviations: *iPGM*, cofactor-independent phosphoglycerate mutase; dPGM, cofactor-dependent PGM; *w*Bm*, Wolbachia* from *Brugia malay*; MBP, maltose binding protein

Résumé

Une phosphoglycérate mutase cofacteur indépendante putative a été identifiée dans le génome de *Wolbachia*, le symbiote intracellulaire de la filaire *Brugia malayi*. Les phosphoglycérates mutases inter convertissent le 2- et 3-phosphoglycerate dans les voies glycolytique et néoglucogénique. Le gène de *Wolbachia* a été cloné avec succès et surexprimé dans la levure *Kluyveromyces lactis* et la protéine purifiée a exhibé une activité de phosphoglycérate mutase typique. La phosphoglycérate mutase cofacteur indépendante n'a pas de similarité de séquence ou structure ave la phosphoglycérate mutase cofacteur dépendante trouvée chez les mammifères, ce qui suggère qu'il puisse être possible de sélectivement cibler l'enzyme du symbiote intracellulaire pour de nouvelles thérapie anti-filaire.

***Mots-clés*:** phosphoglycérate mutase, *Wolbachia*, *Brugia malayi*, glycolyse, néoglucogenèse, filariose

Abstract

A putative cofactor-independent phosphoglycertae mutase gene was identified in the genome sequence of the *Wolbachia* endosymbiont from the filarial nematode, *Brugia malayi*. Phosphoglycerate mutases interconvert 2- and 3-phosphoglycerate in the glycolytic and gluconeogenic pathways. The *Wolbachia* gene was successfully cloned and expressed in the yeast *Kluyveromyces lactis* and the purified protein showed typical phosphoglycerate mutase activity. Cofactor-independent phosphoglycerate mutase has no sequence or structural similarity to the cofactor-dependent phosphoglycerate mutase found in mammals suggesting it may be possible to selectively target the endosymbiont enzyme as a novel anti-filarial therapy.

***Keywords*:** phosphoglycerate mutase, *Wolbachia*, *Brugia malayi*, glycolysis, gluconeogenesis, filariasis

Chapter 2

In recent years obligate α-proteobacterial endosymbionts belonging to the genus *Wolbachia* that are present in most filarial nematode species have become the focus of intense study as a new approach to chemotherapy. Studies performed *in vitro* or in animal models, as well as clinical trials in humans have shown the susceptibility of *Wolbachia* to the tetracycline family of antibiotics. Tetracycline-mediated clearance of *Wolbachia* from nematode tissues correlates with a block in embryogenesis and worm development and, in certain situations, leads to a significant reduction in adult worm burdens and a reduction in associated pathology (Taylor *et al.*, 2005; Hoerauf, 2006). These studies have clearly demonstrated the feasibility of treating filarial infections through anti-*Wolbachia* therapies, but the protracted treatment regimens required for efficacy, coupled with contra-indications for young children and pregnant or breastfeeding women, render tetracycline therapy in its present form unsuitable for mass treatment in endemic areas. Therefore, there is a need to discover alternative anti-*Wolbachia* treatments which may be facilitated by identification of new drug targets in this endosymbiont.

The completed genome sequence of the *Wolbachia* endosymbiont from the filarial nematode *Brugia malayi* (*w*Bm) (Foster *et al.*, 2005) has generated an unrivalled resource for identification of enzymes and processes that are either lacking in mammals or differ substantially from their mammalian counterparts, warranting their further evaluation as candidate drug targets. We have identified a putative cofactor-independent phosphoglycerate mutase (iPGM) in the catalog of proteins predicted by the *w*Bm genome sequence. Phosphoglycerate mutase (PGM) catalyzes the interconversion of 2- and 3-phosphoglycerate (2-PG and 3-PG) in the glycolytic and gluconeogenic pathways but exists in two distinct forms, iPGM and cofactor-dependent phosphoglycerate mutase, dPGM (Fothergill-Gilmore and Watson, 1989; Jedrzejas *et al.*, 2000b). The iPGM proteins are ~ 57 kD monomers and promote the intramolecular transfer of the phosphoryl group between the monophosphoglycerates through a phosphoserine intermediate. Conversely, dPGM is ~ 27 kD although the enzyme is usually active as a dimer or tetramer and catalyzes the intermolecular transfer of the phosphoryl group between the monophosphoglycerates and the cofactor (2,3-diphosphoglycerate) via a

phosphohistidine intermediate. Furthermore, there is no similarity in either the primary sequences or three-dimensional structures of iPGM and dPGM enzymes (Fothergill-Gilmore and Watson, 1989; Jedrzejas *et al.*, 2000b). Vertebrates possess only dPGM (Carreras *et al.*, 1982) which has raised the suggestion that iPGM may represent a potential drug target in pathogenic organisms that contain only that form (Fraser *et al.*, 1999; Galperin and Jedrzejas, 2001; Zhang *et al.*, 2004). Significantly, our searches of the *Wolbachia* genome using either *Escherichia coli* dPGM (GenBank accession no. **BAA35417**) or human dPGM (GenBank accession no. **P15259**) returned no matches indicating iPGM is the only PGM form encoded by the endosymbiont genome. Interestingly, iPGM is also the only PGM present in *B. malayi*, (Zhang *et al.*, 2004) suggesting that both the *Wolbachia* and nematode enzymes could be simultaneously targeted by a single inhibitor.

We undertook the cloning of *w*Bm-iPGM, the cofactor-independent PGM of the *Wolbachia* endosymbiont of the nematode, *B. malayi*. The open reading was amplified from a bacterial artificial chromosome, BMBAC39G04 (Foster *et al.*, 2004), known to contain the gene, using Phusion High-Fidelity DNA Polymerase (New England Biolabs) and the following primers: Forward
5'-GATCTACTCGAGATGAACTTTAAGTCAGTTGTTTTATG-3' (XhoI site underlined) and Reverse
5'- ATAAGAATGCGGCCGCTTACACAATCAGTGAACTACCTGT -3' (NotI site underlined). The PCR product was cloned between the corresponding sites of the vectors, pKLMF-EK and pKLMF-FX (New England Biolabs) for intracellular expression of *w*Bm-iPGM bearing an in-frame N-terminal maltose binding protein (MBP) fusion partner, separated by either an enterokinase (EK) or Factor Xa (FX) protease cleavage site, in the yeast *Kluyveromyces lactis* (New England Biolabs). The integrity of the cloned sequences was confirmed by DNA sequencing.

The sequence of the cloned *wBm-iPGM* (1506 bp) was identical to the gene predicted by the complete *Wolbachia* genome. The deduced protein (~56 kD) contains the catalytic serine and 13 other critical residues indicated by structural analysis of the

biochemically characterized iPGM from *Bacillus stearothermophilus* (Jedrzejas *et al.*, 2000a, b) (Figure S1).

Figure S1: Alignment of the deduced amino acid sequences of various iPGM enzymes.

The sequence of iPGM from the *Wolbachia* endosymbiont of *Brugia malayi* (*w*Bm; GenBank accession no. **AAW70991**) is aligned with the extensively studied and structurally characterized iPGM from *Bacillus stearothermophilus* (Bs; GenBank accession no. **Q9X519**) (Jedrzejas *et al.*, 2000a, b) and the characterized iPGM from *Brugia malayi* (Bm; GenBank accession no. **AAQ97626**) (Zhang *et al.*, 2004; Raverdy *et al.*, 2007). Residues that are identical in at least two of the three sequences are shaded in black, while conserved amino acids are grey. The catalytic serine (@) and 13 other residues (✱) involved in catalysis (Jedrzejas et al., 2000b) are conserved in all three enzymes. Alignment generated with ClustalW and displayed with BOXSHADE (www.ch.embnet.org/software/BOX_form.html).

*w*Bm-iPGM has greatest similarity with the iPGM proteins predicted by the genomes of other α-proteobacteria, notably other rickettsial organisms such as *Anaplasma marginale* (56% identity) and *Ehrlichia* ruminantium (~53%). It also has similarity to the experimentally characterized iPGMs from bacteria such as *Escherichia coli* (Fraser *et al.*, 1999) (44% identity) and *B. stearothermophilus* (Chander *et al.*, 1999) (43%) as well as from certain protozoan parasites such as *Trypanosoma brucei* (Chevalier

et al., 2000; Djikeng et al., 2007) (31%), and from nematodes such as *B. malayi* (Zhang *et al.*, 2004; Raverdy *et al.*, 2007) (~40%). Lateral gene transfer events between *Wolbachia* and their invertebrate hosts, including filarial nematodes, appear widespread (Hotopp *et al.*, 2007) and evidence for gene transfer of iPGM between or within archaea, bacteria and protozoans has been reported (Liapounova *et al.*, 2006; Johnsen and Schonheit, 2007). However, the clustering of *w*Bm-iPGM with other α-proteobacterial iPGM enzymes and a lower amino acid similarity to iPGM from *B. malayi*, which itself clusters with other nematode iPGMs (Zhang *et al.*, 2004; Raverdy *et al.*, 2007) argues against lateral gene transfer being responsible for the presence of iPGM in both *Wolbachia* and its nematode host.

In order to demonstrate that *wBm-iPGM* encodes an active PGM, recombinant enzyme was produced for biochemical studies. Attempts to express *w*Bm-iPGM in *E. coli* were unsuccessful despite using different expression systems that produced protein with either a N-terminal MBP tag, a C-terminal His_6 tag, or untagged protein produced by intein-mediated cleavage of a chitin binding domain fusion partner. In these systems, *w*Bm-iPGM was mostly insoluble and the small amounts of soluble protein obtained were relatively impure and inactive (data not shown). These results were surprising given that we and others have produced several active iPGM enzymes from diverse organisms in these bacterial expression systems (Fraser *et al.*, 1999; Collet *et al.*, 2001; Guerra *et al.*, 2004; Zhang *et al.*, 2004; Djikeng *et al.*, 2007; Raverdy *et al.*, 2007). Unlike other expressed iPGM enzymes, *w*Bm-iPGM has a high number of cysteine residues and the formation of disulfide bonds is strongly predicted (http://contact.ics.uci.edu/bridge.html). However, there was no difference in expression and activity of *w*Bm-iPGM when produced in *E. coli* strains having either reducing or oxidizing cytoplasms. Similarly, purification in the presence of reducing agents (DTT or β-mercaptoethanol) did not yield active protein. Expression of a synthetic *wBm-iPGM* gene, optimized for *E. coli* codon usage, also failed to improve expression. Active His-tagged *w*Bm-iPGM was finally recovered from *E. coli* by urea denaturation of insoluble protein and subsequent refolding, but the specific activity of the refolded protein was only 0.04 units/mg (data not shown).

The difficulties of producing active recombinant *w*Bm-iPGM in *E. coli* prompted us to attempt expression in yeast cells. We successfully produced *w*Bm-iPGM as a MBP fusion in *Kluyveromyces lactis*. The MBP moiety serves as both a tag for purification and an aid to solubility (Kapust and Waugh, 1999). The construct, *pKLMF-EK-wBm-iPGM*, was linearized with SacII and used to transform *K. lactis* GG799 competent cells following the *K. lactis* Protein Expression Kit recommendations (New England Biolabs). Transformants containing multiply-integrated copies of the expression cassette, which usually produce more recombinant protein, were identified by PCR of patched colonies using the kit's Integration Primer 3 coupled with the following primer: 5'-GTTTACCTTCTTCAGTTTTCAT-3' and selected for production of *w*Bm-iPGM. A single colony was used to inoculate 3 ml YPGlucose (10 g yeast extract, 20 g Bacto Peptone/L, 2% glucose) and cells were grown with shaking at 240 rpm at 30^{o}C overnight. Two 1L YPGalactose (2%) cultures were inoculated with 1 ml each of the overnight culture, and cells were grown in baffled flasks at 30^{o}C with shaking at 240 rpm for 3 days. Cells were pelleted at 6000 x g for 15 min at 4^{o}C and pooled to give about 24 g wet cells. The cells were washed once in water then resuspended in 25 ml Resuspension buffer (1M Sorbitol, 50 mM Tris-HCl, pH 7.5, 30 mM DTT, 5% Glycerol) supplemented with 1 Complete EDTA-free protease inhibitor cocktail tablet (Roche Applied Sciences) and incubated for 10 minutes at 30^{o}C. The cells were collected at 6000 x g for 5 min at 4^{o}C and resuspended in 25 ml Breakage buffer (1M Sorbitol, 50 mM Tris-HCl, pH 7.5, 2 mM DTT, 5% Glycerol) supplemented with 1 Complete EDTA-free protease inhibitor cocktail tablet. A 10 mg/ml stock of Zymolase 20T (Associates of Cape Cod Inc.) in 30 mM Sodium Phosphate buffer, pH 7.0, was added to a final concentration of 0.5 mg/ml. The cells were incubated at 30^{o}C for 30 min with occasional gentle mixing. Spheroplasts were collected by centrifugation and washed twice in Breakage buffer to remove the Zymolase 20T. The pellet was resuspended in 4 volumes (~100 ml) of ice-cold Column buffer (20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1 mM DTT, 5% Glycerol) supplemented with 4 Complete EDTA-free protease inhibitor cocktail tablets. The cell suspension was passed twice through a TS Series Bench Top Cell Disruptor (Constant Systems,) at 40 Kpsi. The sample was centrifuged at 14,000 x g for 30 min at 4^{o}C and

the supernatant harvested. The cell extract was loaded on to a 10 ml amylose column according to the pMAL Protein Fusion and Purification System recommendations (New England Biolabs). Recombinant *w*Bm-iPGM was eluted with column buffer containing 10 mM maltose and 1.5 ml fractions were collected for separation by SDS-PAGE.

*w*Bm-iPGM was produced with a high degree of purity (Figure 1). The apparent molecular weight (~100 kDa) is consistent with the calculated size of iPGM (~58 kDa) fused to MBP (~42 kDa). The identity of this ~100 kDa protein as a MBP fusion was confirmed by Western blot using an anti-MBP monoclonal antibody (New England Biolabs; data not shown).

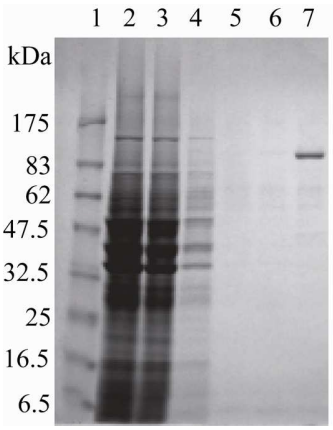

Figure 1: Purification of *w*Bm-iPGM expressed intracellularly in the yeast *K. lactis*.

Lane 1, Prestained Protein marker, Broad range (NEB); lane 2, *K. lactis* lysate; lane 3, flow-through from amylose column; lane 4, column wash; lanes 5, 6 and 7, elution fractions.

The activity of the *w*Bm-iPGM fusion protein was measured in the forward (glycolytic) direction using an established enzyme-coupled assay (Raverdy *et al.*, 2007) in which PGM activity is determined indirectly by monitoring the consumption of NADH at 340 nm. A typical PGM activity was observed (Figure 2) and from the slope of the curve a specific activity of 4.1 units/mg was calculated. One unit of PGM activity is defined as the amount of activity that is required for the conversion of 1.0 μmole NADH to NAD per minute.

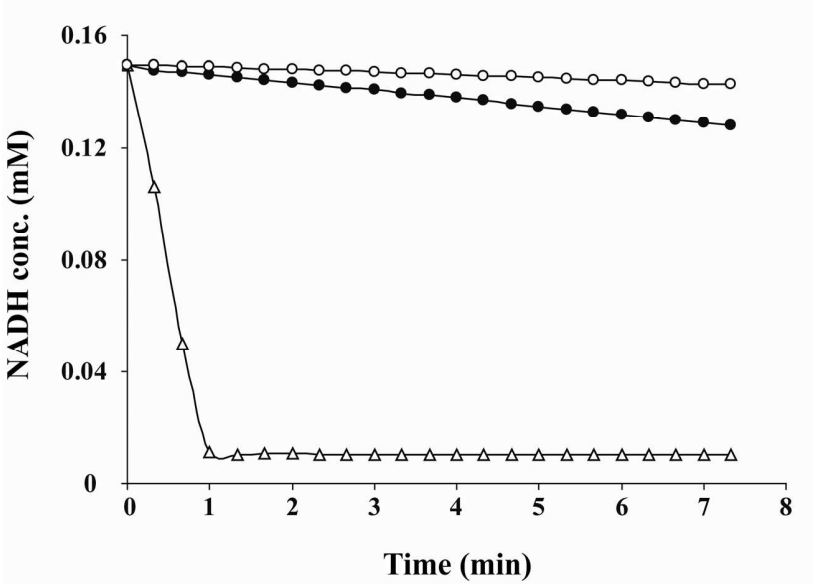

Figure 2: Activity of recombinant *w*Bm-iPGM.

Conversion of 3-PG to 2-PG by *w*Bm-iPGM (λ) is measured indirectly by a decrease in NADH concentration, determined spectrophotometrically at 340 nm. Consumption of NADH is directly proportional to PGM activity. A negative control lacking *w*Bm-iPGM (○) and a positive control using 2-PG to demonstrate activity of the coupling enzymes (△) are shown.

The specific activity of *w*Bm-iPGM produced in *K. lactis* was ~100-fold higher than that obtained in *E. coli* after denaturation and refolding of insoluble protein and is broadly similar to activities reported for other bacterial iPGM enzymes (Kuhn *et al.*, 1993; Leyva-Vazquez and Setlow, 1994; Chander *et al.*, 1999) but lower than most eukaryotic iPGMs where specific activities are typically in the range of 50 to 400 units/mg (Chevalier *et al.*, 2000; Guerra *et al.*, 2004; Zhang *et al.*, 2004). iPGMs are metalloenzymes and all characterized bacterial enzymes appear to use manganese as the preferred ion (Jedrzejas and Setlow, 2001). We did not observe any enhancement in *w*Bm-iPGM activity when the standard magnesium-containing buffer was supplemented with cobalt, manganese or zinc. Thus, the preferred metal ion of *w*Bm-iPGM is still unknown.

The essential roles of *Wolbachia* endosymbionts in filarial nematode biology have resulted in these bacteria being considered as an Achilles' heel of their worm hosts and

proof of principle clinical trials using tetracycline antibiotics are testament to that view (Taylor *et al.*, 2005; Hoerauf, 2006). We have initiated studies to identify and characterize new candidate drug targets predicted by the *w*Bm genome sequence. The identification of *w*Bm-iPGM and production of active recombinant protein for further studies represents one of the first examples of utilizing the *Wolbachia* genome to facilitate novel strategies for filarial disease control by targeting the *Wolbachia* endosymbiont. The lack of similarity in primary sequence or tertiary structure between dPGM and iPGM makes it highly probable that an iPGM inhibitor would not affect the mammalian enzyme. The likelihood that iPGM inhibition would disrupt filarial biology either directly or through targeting the *Wolbachia* endosymbiont appears high. In all organisms where iPGM is the only form of PGM present and the gene has been deleted or its transcript level reduced by RNAi, deleterious effects have been observed. Reduction of iPGM activity in *C. elegans* by RNAi resulted in embryonic lethality, larval lethality and abnormal morphology (Zhang *et al.*, 2004). An iPGM gene deletion in the bacterial species *Bacillus subtilis* resulted in severely slowed growth and inability to sporulate (Leyva-Vazquez and Setlow, 1994) while in *Pseudomonas syringae* an iPGM deletion lead to failure to grow and an inability to establish infection on host plants (Morris *et al.*, 1995). These studies in nematodes and bacteria demonstrate the feasibility of developing novel therapies that target the iPGM of *B. malayi* or its *Wolbachia* endosymbiont.

That iPGM is present in *Wolbachia*, nematodes and several other pathogens while absent in mammals makes it an attractive target in diverse organisms. Therefore, identification of an iPGM inhibitor could lead to new therapeutic strategies not only for filarial disease control but additionally for a range of infectious diseases. The availability of active recombinant iPGM enables further development of this target and its inclusion in high-throughput inhibitor screens.

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Chapter 3: Cofactor-independent phosphoglycerate mutase is an essential gene in procyclic form *Trypanosoma brucei*

La phosphoglycérate mutase cofacteur indépendante est un gène essentiel de *Trypanosoma brucei* procyclique

Cet article décrit une étude sur la phosphoglycérate mutase cofacteur indépendante du parasite *Trypanosoma brucei* effectuée en collaboration avec des scientifiques de l'Institut de recherche génomique (TIGR). *T. brucei* est une des espèces de trypanosomes responsable de la maladie du sommeil chez l'homme. Les médicaments utilisés pour traiter cette maladie sont inadéquats et des efforts considérables sont fait pour identifier de nouvelles cibles thérapeutiques. Notre laboratoire a noté que la PGMi était présente dans un certains nombre de pathogènes humains, dont *T. brucei*, et nous étions intéressés dans la poursuite de la PGMi en tant que cible thérapeutique à large spectre. Il est important de noter que l'interférence à ARN (RNAi) est bien établie chez *T. brucei*, nous permettant ainsi de déterminer directement si l'enzyme est requise pour le développement du parasite et ainsi de la valider en tant que cible thérapeutique. Nous avons choisi d'étudier la forme procyclique de *T. brucei* car le métabolisme énergétique à ce stade est le plus similaire de celui ces autres trypanosomides humains *Leishmania major* et *Trypanosoma cruzi*. Tout d'abord, j'ai cloné et séquencé le gène de *T. brucei* de la souche 927/4 afin de vérifier la séquence du gène puisque nous avons trouvé une divergence entre la séquence prédite du génome et celle reportée auparavant. Par la suite, j'ai produit l'enzyme recombinante en utilisant deux systèmes d'expression différents : le système His-tag et le système IMPACT (sans tag). Les enzymes de nématodes produites dans le laboratoire avaient été produites avec le système His-tag. J'ai pu démontrer que la séquence prédite encodait effectivement une PGMi de *T. brucei* active et que le tag n'influençait pas l'activité. Nos collaborateurs à TIGR ont construit des lignées cellulaires pour effectuer les expériences d'ARN interférence. J'ai évalué l'activité enzymatique endogène dans des lysats préparés à partir de parasites sauvages et des lignées cellulaires dans lesquelles les niveaux d'ARN messagers était sous régulés par ARN interférence. Nous avons démontré que la diminution des ARN messagers de la PGMi à l'équilibre était en corrélation avec une réduction de l'activité enzymatique et un déclin dans la croissance du parasite. Les résultats présentés dans cette publication

indiquent que la PGMi est digne de plus amples recherches en tant que cible thérapeutique chez les trypanosomes.

Cofactor-independent Phosphoglycerate Mutase is an essential gene in procyclic form *Trypanosoma brucei*

This paper described a study on cofactor-independent phosphoglycerate mutase from the parasite *Trypanosoma brucei* performed in collaboration with scientists from The Institute for Genomic Research (TIGR). *T. brucei* is one of the trypanosome species responsible for African trypanosomiasis or sleeping sickness in humans. The drugs used to treat this disease are inadequate and there is considerable effort to identify new drug targets. Our laboratory had noted that iPGM is present in a number of human pathogens, including *T. brucei*, and we were interested in further pursuing iPGM as a 'broad-spectrum' parasite target. Importantly, RNAi is well established in *T. brucei*, enabling us to determine directly if the enzyme is required for development and therefore validating it as a drug target. We chose to study procyclic (insect) form *T. brucei* since energy metabolism in this stage is more similar to that found in the other important human trypanosomatid parasites *Leishmania major* and *Trypanosoma cruzi*. Firstly, I cloned and sequenced the gene from the *T. brucei* strain 927/4 in order to verify the gene sequence since we found a discrepancy between the sequence predicted from the genome sequence and a previous report. Subsequently, I produced recombinant protein using two different expression systems, the His-tag and Impact (no tag) systems. The nematode iPGM enzymes studied in the lab had been produced using the His-tag system. I demonstrated that the predicted iPGM sequence did indeed encode an active *T. brucei* iPGM and found that the presence of a His-tag did not alter this activity. Our collaborators at TIGR constructed cell lines to perform RNAi experiments. I evaluated the levels of endogenous enzyme activity in lysates prepared from wild-type parasites and cell lines in which the mRNA levels were down-regulated by RNAi. We demonstrated that depletion of the steady state levels of iPGM mRNA correlated with a reduction in enzyme activity and a decline in the growth of the parasite. The results in this publication indicate that iPGM is worthy of further investigation as a drug target in trypanosomes.

Chapter 3

Cofactor-independent Phosphoglycerate Mutase is an essential gene in procyclic form *Trypanosoma brucei*

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***Running title*:** RNAi of *Trypanosoma brucei* cofactor-independent Phosphoglycerate Mutase.

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***Abbreviations*:** dsRNA, double stranded RNA; RNAi, RNA interference; iPGAM, co-factor-independent phosphoglycerate mutase; dPGAM, cofactor-dependent phosphoglycerate mutase; 2-PG and 3-PG, 2- and 3-phosphoglycerate.

Résumé

La glycolyse et la néoglucogenèse sont en partie contrôlées par l'inter conversion du 3- et 2-phosphoglycérate (3-PG et 2-PG) qui est réalisée par les phosphoglycérates mutases (PGAMs). Ces dernières peuvent être dépendante d'un cofacteur (PGAMd) ou non (PGAMi). Le trypanosome africain, *Trypanosoma brucei*, possède la forme indépendante PGAMi, laquelle est supposée jouer un rôle important dans la glycolyse. Nous rapportons ici, l'utilisation de l'interférence ARN pour sous-réguler le gène de la PGMi chez *T. brucei,* au stade procyclique et l'évaluation le phénotype résultant. Nous avons tout d'abord démontré de façon biochimique que la diminution du niveau des ARN messagers de PGAMi à l'équilibre est en corrélation avec la réduction flagrante de l'activité enzymatique. Nous montrons ensuite que la PGAMi est requise pour la croissance cellulaire des *T. brucei* procycliques.

Mots-clés : *Trypanosoma brucei*, glycolyse, phosphoglycérate mutase, ARNi

Abstract

Glycolysis and gluconeogenesis are, in part, driven by the interconversion of 3- and 2-phosphoglycerate (3-PG and 2-PG) which is performed by phosphoglycerate mutases (PGAMs) which can be cofactor dependant (dPGAM) or cofactor independent (iPGAM). The African trypanosome, *Trypanosoma brucei*, possesses the iPGAM form which is thought to play an important role in glycolysis. Here, we report on the use of RNA interference to down-regulate the *T. brucei* iPGAM in procyclic form *T. brucei* and evaluation of the resulting phenotype. We first demonstrated biochemically that depletion of the steady state levels of iPGAM mRNA correlates with a marked reduction of enzyme activity. We further show that iPGAM is required for cell growth in procyclic *T. brucei*.

Keywords**:** *Trypanosoma brucei*, glycolysis, phosphoglycerate mutase, RNAi.

Chapter 3

The disease, African trypanosomiasis, caused by *Trypanosoma brucei* and other related trypanosome species (such as *Trypanosoma congolense*, *Trypanosoma equiperdum*, *Trypanosoma vivax*) poses serious human and animal health problems in most parts of Africa and beyond (Barrett *et al.*, 2003; Joshi *et al.*, 2005). Control methods are still unreliable and there is an increasing need for new and more effective therapies (Jannin and Cattand, 2004). There has been a considerable focus on *T. brucei* as a "model" trypanosomatid to understand important aspects of the biology of these parasites which may lead to the identification of potential drug targets and other reagents for a more effective control of trypanosomiasis and leishmaniasis.

Energy metabolism in *T. brucei* differs significantly from that of its mammalian host with distinct enzymes and pathways present in the parasite (Hannaert *et al.*, 2003). In addition, some of the glycolytic enzymes are localized in unique organelles called glycosomes which are known to be essential for *T. brucei* (Guerra-Giraldez *et al.*, 2002) and probably for other trypanosomatids as well. Therefore, various components and steps of the glycolysis pathway have been intensely studied to further understand how this parasite acquires/regulates its energy, and pursued as possible targets for drug development (Albert *et al.*, 2005; Besteiro *et al.*, 2005). During glycolysis and gluconeogenesis, the interconversion of 3- and 2-phosphoglycerate (3-PG and 2-PG) is performed by phosphoglycerate mutases (PGAMs). In nature, two unrelated types of PGAM exist that are either cofactor (2, 3-diphosphoglycerate)-dependent (dPGAM) or cofactor-independent (iPGAM). Importantly, the 2 enzymes do not share any sequence or structural similarity, and operate via different catalytic mechanisms (Jedrzejas *et al.*, 2000b, a; Rigden *et al.*, 2002). A PGAM activity has been demonstrated in *T. brucei* lysates (Oduro *et al.*, 1980) and it was subsequently determined that the parasite possesses the iPGAM form of PGAM (Chevalier *et al.*, 2000; Collet *et al.*, 2001). The presence of iPGAM in *T. brucei*, and the knowledge that all vertebrates possess only dPGAM, opens a possibility for selective inhibition of iPGAM as an approach to new drug discovery efforts for trypanosomiasis (Chevalier *et al.*, 2000).

The genome sequence of *T. brucei* strain 927/4 (Berriman *et al.*, 2005) predicted an iPGAM sequence (Locus name: Tb10.6k15.2620 and GenBank Accession number:

XM_817934) that encodes a protein having a region of 25 amino acids near the C-terminus that is highly divergent from a *T. brucei* iPGAM described earlier (Chevalier *et al.*, 2000). These 25 aa, beginning at position 501 of the predicted *T. brucei* iPGAM have almost 100% identity with both the published *Leishmania mexicana* iPGAM (Guerra *et al.*, 2004) and with the *Trypanosoma cruzi* iPGAM predicted in the complete *T. cruzi* genome sequence (El-Sayed *et al.*, 2005a). However, the *T. brucei* iPGAM sequence reported earlier (Chevalier *et al.*, 2000) has no similarity in this region to the other trypanosomatid iPGAM sequences. To confirm that the predicted gene indeed encodes a functional iPGAM, we cloned and expressed the gene as a tag-free recombinant protein by using the IMPACT system (New England Biolabs) and with an N-terminal 6xHis Tag using the pET28 vector (Novagen) according to the manufacturer's instructions. The recombinant protein was ~60 kDa in size as predicted from the sequence (Figure 1, data for tag-free protein shown) and possessed iPGAM activity when assayed in the glycolytic direction as described (Zhang *et al.*, 2004).

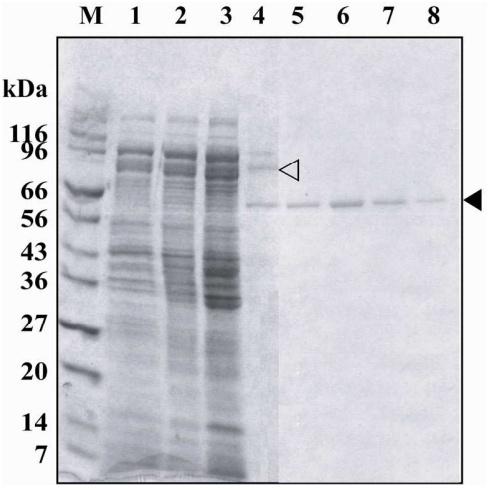

Figure 1: Purification of recombinant *T. brucei* iPGAM.

The predicted *Tb-iPGAM* (Tb10.6k15.2620) gene was amplified from genomic DNA using the following primers: Forward: 5' atg cac cat atg atg gca ctc acg ctt gct 3' (*Nde*I site underlined); Reverse: 5' gcg cat act agt cat tac aat ggt gtc acc gga tac gca cgc tac ctc aat gag cga cgg t 3' (*Spe*I site underlined). The resulting PCR product (~1700 bp) was digested with *Nde*I and *Spe*I (New England Biolabs, NEB) and cloned into the corresponding sites of the pTWIN vector (NEB). Recombinant plasmids were sequenced to ensure authenticity of the insert. *Escherichia coli* cultures were grown in LB medium and expression was induced with 0.1 mM isopropyl-1-thio-ß-D-galactopyranoside (IPTG) overnight at 15°C. The recombinant protein was purified according to the manufacturer's instructions (NEB). Protein samples were electrophoresed on a 4-20% gradient SDS-PAGE gel. *M*: protein marker (NEB). *Lane 1*: *E. coli* total extract before induction; *lane 2*: soluble *E. coli* protein following induction with IPTG; *lane 3*: pellet; *lane 4*: sample of the column resin prior to the cleavage of fusion partner and elution; *lanes 5-8*: consecutive elution fractions after overnight cleavage by 40 mM DTT at 4°C. *Filled arrowhead* indicates the purified *T. brucei* iPGAM, *open arrowhead* indicates the intein/iPGAM fusion protein before cleavage.

The specific activities (one unit is defined as the number of micromoles of substrate converted per minute) with a 6xHis tag (14 units/mg) and without any tag (27 units/mg) were comparable. These levels of specific activities were similar to that previously reported for a 6xHis tagged *Tb*-iPGAM (Collet *et al.*, 2001) but lower than that described in another study (Chevalier *et al.*, 2000) or for *L. mexicana* iPGAM (Guerra *et al.*, 2004).

The life cycle of *T. brucei* takes place in the mammalian host and in the insect vector. In the mammalian host, the parasite dwells in the bloodstream where it relies on energy obtained exclusively from glucose via glycolysis (Albert *et al.*, 2005) . In contrast, in the insect stage (procyclic form) of *T. brucei* amino acids can be used as an energy source when glucose is in limited supply (Cross *et al.*, 1975; Lamour *et al.*) . The more elaborate energy metabolism of *T. brucei* procyclic forms is also found in other trypanosomatids such as *T. cruzi* and *Leishmania* spp (Besteiro *et al.*, 2005). On this basis, it was recently proposed that results of studies of energy metabolism conducted in the procyclic form of *T. brucei* can be extrapolated to understand the situation in other human pathogenic trypanosomatids (Hannaert *et al.*, 2003; Besteiro *et al.*, 2005).

To determine if iPGAM is an essential enzyme in the procyclic form of *T. brucei* when the parasites are grown in a glucose-containing medium (Cunningham medium (Cunningham, 1977), Hyclone), which contains 0.7 g/L glucose, supplemented with 10% fetal bovine serum), we used dsRNA induced RNA interference (RNAi) to down-regulate the corresponding mRNA aiming at consequently down-regulating its endogenous activities. We first constructed RNAi cell lines for conditional targeting and degradation of iPGAM mRNA levels. To this end, we polymerase chain reaction (PCR)-amplified and cloned a DNA fragment corresponding to nucleotides 1-699 of the *Tb-iPGAM* ORF into the pZJM vector that allows the expression of dsRNA under the control of two opposing tetracycline inducible T7 promoters (Wang *et al.*, 2000). The recombinant RNAi plasmid, termed pZJM-iPGAM, was digested and used to transfect 29.13.6 procyclic form trypanosomes using standard protocols (Djikeng *et al.*, 2004). The resulting stable transformant cells were selected using antibiotics added to the culture

medium (see legend of Figure 2a for details), cloned by limiting dilution and individual clones were selected for further analysis. We next experimentally established the knockdown effect of RNAi by simultaneously monitoring the expression of dsRNA and the steady state levels of iPGAM mRNA. We induced 4 different clonal cell lines (Tb-iPGAMRNAi-9.2, Tb-iPGAMRNAi-10.2, Tb-iPGAMRNAi-13.1 and Tb-iPGAMRNAi-13.2) and determined the levels of induced dsRNA and endogenous full-length iPGAM by Northern Blot analysis (Figure 2a). After addition of tetracycline into the culture medium for the induction of the two T7 promoters for 48 hours, expression of iPGAM dsRNA is evidenced by the presence of an intense RNA band of ~700 bp in contrast to non-induced samples, coinciding with the observed almost complete reduction of the steady state levels of *Tb*-iPGAM mRNA.

We next sought to evaluate the effect of a reduction in *Tb*-iPGAM mRNA levels on endogenous enzyme activity. To this end, we selected for induction, two representative clonal cell lines that showed a significant degradation of *Tb*-iPGAM mRNA upon induction for expression of the cognate dsRNA (Tb-iPGAMRNAi-9.2 and Tb-iPGAMRNAi-10.2) for 48 h since iPGAM mRNA levels were reduced to a very low level at this time point (Figure 2a). The cells were then harvested, and extracts were prepared and assayed for iPGAM activity as previously described (Oduro *et al.*, 1980; Chevalier *et al.*, 2000; Zhang *et al.*, 2004). A similar extract was also prepared from the same number of non-induced control cells. A significant decrease (~fivefold) in iPGAM activity was observed in both cell lines compared to non-induced control cells (Figure 2b, data for Tb-iPGAMRNAi-9.2 shown). The discrepancy between the observed residual enzyme activity and the almost complete disappearance of the mRNA can be explained by the difference of turn over of the mRNA and its corresponding protein. In general, in most RNAi experiments, the reduction of the mRNA levels almost always precede those of the corresponding protein.

To determine if the observed down-regulation in iPGAM mRNA and enzyme activity levels have an effect on growth of procyclic form *T. brucei* cells, Tb-iPGAMRNAi-9.2 clonal cells were grown in the presence or in the absence of tetracycline added daily to culture medium for 8 days (Figure 2c). The number of

parasites was determined on a daily basis for both induced and non-induced cells. By day 4, we observed a decline in the rate of cell growth in induced as compared to non-induced cells. The decline in growth rate most likely indicates that *Tb*-iPGAM is required for normal proliferation of procyclic *T. brucei*. No other morphological defect of the slow growing induced cells was detected.

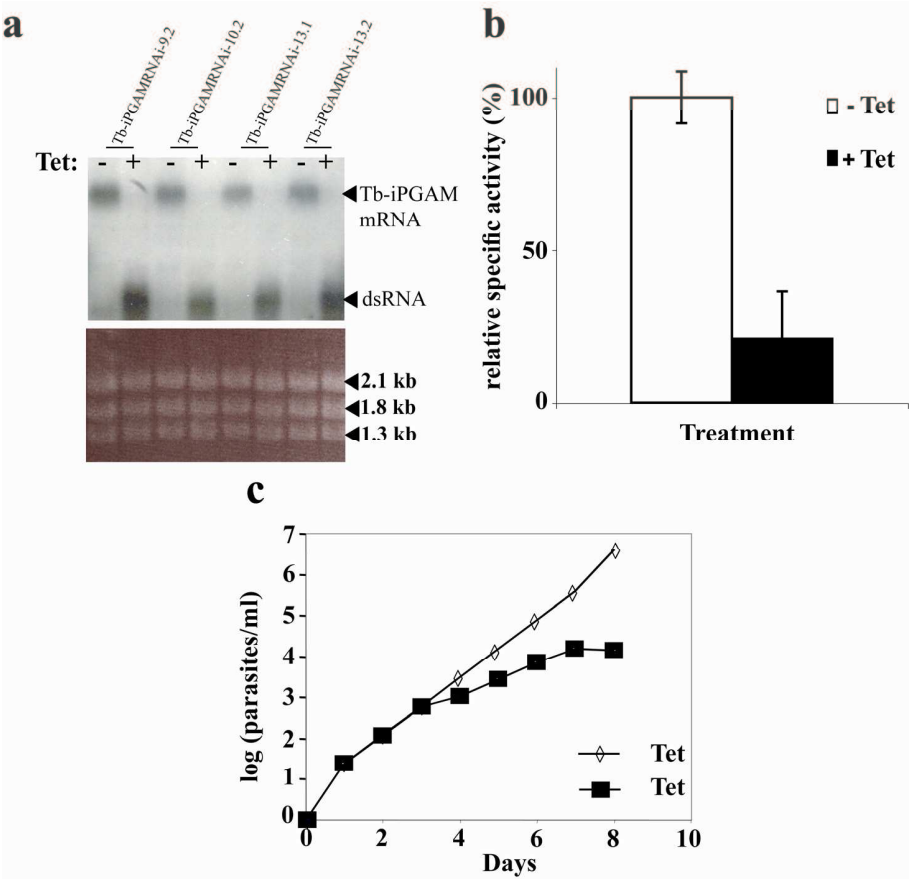

Figure 2: a. Down regulation of *Tb*-iPGAM steady state mRNA levels. Northern Blot analysis (*upper panel*) of total RNA prepared from 4 different (tet)-induced and non-induced *Tb*-iPGAM RNAi cell lines.

To generate the *Tb*-iPGAM RNAi cell lines (Tb-iPGAMRNAi), we PCR-amplified a 699-nt DNA fragment of the *Tb-iPGAM* ORF (1 – 699 nt) using the following oligonucleotides each containing *Xba*I restriction sites: Forward: 5' gc tct aga atg gca ctc acg ctt gct gct cac aag 3'; Reverse: 5' gc tct aga cga ttt gaa ggc gcg tcc ctc acc aag cac 3' (the *Xba*I restrictions sites are *underlined*). The PCR product was digested with *Xba*I and cloned between the tetracycline (tet) inducible T7 promoters of the pZJM RNAi plasmid (Wang *et al.*, 2000) digested with *Xba*I. The recombinant plasmid pZJM-iPGAM was linearized

with *Not*I to allow the integration into the rDNA non-transcribed spacer of procyclic form *T. brucei* cells clone 29-13-16 that expresses both the tet repressor and the T7 RNA polymerase (Wirtz *et al.*, 1999). For transfection, 30μg of linearized pZJM-iPGAM was used for $1x10^8$ cells. Transfected cells were selected in Cunningham medium (Cunningham, 1977) in the presence of the following antibiotics: phleomycin (2.5μg/ml), G418 (15μg/ml), hygromycin (50μg/ml). A population of stable transformants was established after 10 days of selection and then cloned by limiting dilution. Four clones (Tb-iPGAMRNAi-9.2, Tb-iPGAMRNAi-10.2, Tb-iPGAMRNAi-13.1 and Tb-iPGAMRNAi-13.2) were selected for further analysis. Selected clones were induced for 48 hours by addition into the culture medium of tetracycline dissolved in 70% ethanol to a give a final concentration of 10 μg/ml. Control cells received identical volumes of 70% ethanol. After 48 hours, $1x10^8$ induced and non-induced control cells were pelleted, washed twice (with a cell wash solution containing: 100mM NaCl, 20mM Tris-Cl pH 7.5, 3mM $MgCl_2$) and the pellet resuspended with 1 ml of Trizol (Invitrogen). Total RNA was prepared following the manufacturer's instructions. To confirm the expression of dsRNA and degradation of the cognate mRNA, 10μg of total RNA from each sample was resolved in a 1.2% agarose – 2.2M formaldehyde gel, transferred onto nitrocellulose membrane (uncharged Hybond, Amersham Biosciences). The membrane was hybridized using a ^{32}P-labeled DNA fragment representing the entire *Tb-iPGAM* ORF. To visualize loading amount of total RNA samples, the gel was stained using ethidium bromide and photographed using the Biorad gel documentation system (lower panel). *Arrows* indicate rRNA bands. Pre-hybridizations and hybridization were performed as previously reported (Djikeng *et al.*, 2004).

b Measurement of endogenous PGAM activity in induced and non-induced Tb-iPGAMRNAi-9.2 cells.

Cells were induced by addition of tetracycline (+ tet) into the culture medium for 48 hours, harvested and processed for total protein lysates as described (Oduro *et al.*, 1980; Chevalier *et al.*, 2000). A similar extract was also prepared from the same number of non-induced, control cells (- tet). Extracts were then assayed in a standard coupled-enzyme reaction (Zhang *et al.*, 2004) in the presence and absence of substrate to determine activity specifically due to iPGAM.

c Effect of RNAi on the growth curve of induced (+ tet, *solid symbol*) and non-induced (- tet, *open symbol*) Tb-iPGAMRNAi-9.2 cells.

Cells were grown to a density of $1x10^7$ cells/ml, diluted to $1x10^6$ cells/ml and divided into two flasks. Each flask received either 10 μg/ml of tetracycline or the same volume of 70% ethanol. Cells were diluted and counted each day over the course of 8 days. The growth curve was graphed by using the log of cell density (shown on the *y*-axis) versus day of culture.

In summary, our results demonstrate that iPGAM is required for the normal growth of procyclic *T.brucei* in a glucose-containing medium commonly used to maintain the parasites. This is consistent with other data indicating the importance of glycolytic enzymes in procyclic forms grown under similar conditions. It has been shown that knockdown of phosphofructokinase (Kessler and Parsons, 2005) or pyruvate kinase (Coustou *et al.*, 2003) using RNAi is lethal and inhibition of phosphoglycerate kinase using tubericidin results in killing of procyclics T. brucei (Drew *et al.*, 2003). Together these results demonstrate the importance of glycolysis in this stage when glucose is present as occurs in nature following each blood meal in the tsetse fly. RNAi of iPGAM for the bloodstream form of *T. brucei* iPGAM was also found to severely affect growth (Albert *et al.*, 2005), perhaps even more drastically which may be the result of complete dependence on glycolysis for energy generation. Interestingly, iPGAM is known to be important for the growth of certain bacteria, namely, the tomato pathogen *Pseudomonas syringae* (Morris *et al.*, 1995) and *Bacillus subtilis* (Leyva-Vazquez and Setlow, 1994).

In addition, RNAi studies performed in *Caenorhabditis elegans* have also demonstrated the essential nature of iPGAM in nematodes (Zhang *et al.*, 2004). Highly similar iPGAM sequences can be identified in the completed or ongoing genome projects for *T. cruzi* (El-Sayed *et al.*, 2005a), *T, congolense*, *T. vivax*, *T. gambiense*, *L. major* (Ivens *et al.*, 2005), *Leishmania infantum*, and *Leishmania braziliensis,* and have been reported earlier for *L. mexicana* (Guerra *et al.*, 2004). The comparative analysis of the genomes of three kinetoplastids, *T. brucei*, *T. cruzi* and *L. major,* identified a group of 6,200 genes now referred to as the "Tritryp core proteome" which is restricted to these parasites and not found in their mammalian hosts (El-Sayed *et al.*, 2005b). Interestingly, iPGAM is part of the "Tritryp core proteome". It is now generally thought that the "Tritryp core proteome" contains proteins that control essential and parasite-specific biological processes that may be important for their survival and so, represent excellent candidates for new drug development. In this context, we speculate, based on our results using the procyclic form of *T. brucei* as a model for other trypanosomatids that iPGAM may also represent a drug target in *T. cruzi* and *L. major*, the causative agents of South American trypanosomiasis and leishmaniasis.

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Chapter 4: The *Wolbachia* endosymbiont of *Brugia malayi* has an active pyruvate phosphate dikinase

Le symbiote *Wolbachia* de *Brugia malayi* possède une pyruvate phosphate dikinase

Le génome du symbiote *Wolbachia* de *Brugia malayi* (*w*Bm) a été récemment séquencé. Nous avons effectué une analyse des gènes encodants les enzymes de la glycolyse et néoglucogenèse et découvert que *Wolbachia* n'a pas l'enzyme pyruvate kinase responsable dans la majeur partie des organismes (mammifères compris) de la conversion du phosphoénolpyruvate en pyruvate dans la dernière étape de la glycolyse. Cependant, nous avons identifié une pyruvate phosphate dikinase (PPDK) putative qui est capable de catalyser la même réaction. PPDK n'est pas présente chez les mammifères mais a été identifiée dans un petit nombre de pathogènes pour lesquels PPDK a été proposée comme possible cible thérapeutique. Notre but a été de cloner et exprimer la *w*Bm-PPDK putative afin de vérifier son activité et son rôle possible dans le metabolisme du symbiote. D'après les résultats reportés dans la littérature, nous avons anticipé que la protéine recombinante pourrait être difficile à produire et qu'elle pourrait avoir des problèmes de stabilité. J'ai effectivement rencontre de tels problèmes et un certains nombres d'expériences d'optimisation ont été effectuées pour produire la *w*Bm-PPDK sous forme active et stable. Plusieurs souches d'*Escherichia coli*, ainsi que des conditions de cultures variées on été explorées pour obtenir une protéine soluble. Après avoir déterminé les conditions optimales d'expression, un protocole de purification fut établi pour générer un rendement de protéine décent. Le manque de stabilité fut le plus grand problème. Cependant, après avoir essayé plusieurs protocoles utilisant une variété de tampons, j'ai réussi à améliorer la stabilité de l'enzyme en ajoutant un agent réducteur (β-mercaptoethanol) ainsi que du glycérol pour produire une grande quantité d'enzyme active et pure. Nos résultats montrent que *Wolbachia* de *Brugia malayi* possède une pyruvate phosphate dikinase active et cela supporte le développement de cette enzyme en tant que potentielle cible thérapeutique chez les filaires.

The *Wolbachia* endosymbiont of *Brugia malayi* has an active pyruvate phosphate dikinase

The genome of the *Wolbachia* endosymbiont of *Brugia malayi (w*Bm) has been recently sequenced. We performed an analysis of the genes encoding enzymes involved in glycolysis and gluconeogenesis and discovered that *Wolbachia* lacks the enzyme pyruvate kinase responsible in most organisms (including mammals), for the conversion of phosphoenolpyruvate to pyruvate in the last step of the glycolysis. However we identified a putative pyruvate phosphate dikinase (PPDK) which is capable of catalyzing the same reaction. PPDK is not found in mammals but has been identified in a small number of pathogens where it has been proposed as a possible drug a target. Our goal was to clone and express the putative *w*Bm-PPDK protein in order to verify its activity and possible role in the energy metabolism of the endosymbiont. Based on reports in the literature, we anticipated that the recombinant enzyme might be difficult to produce and may have stability issues. I actually did encounter these problems and a number of optimization experiments were performed in order to produce *w*Bm-PPDK in an active and stable form. Several strains of *Escherichia coli*, as well as various culture conditions were investigated to obtain soluble protein. After the optimal expression conditions were determined, a purification protocol was established to generate a decent yield of protein. Lack of stability was the most challenging problem. However after exploring a number of protocols using a variety of different buffers, I managed to improve the stability by adding a reducing agent (β-mercaptoethanol) and glycerol to produce a high yield of highly purified active enzyme. Our results show that *Wolbachia* from *B. malayi* has an active pyruvate phosphate dikinase and support development of the enzyme as a potential drug target in filarial nematodes.

The *Wolbachia* endosymbiont of *Brugia malayi* has an active pyruvate phosphate dikinase

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***Abbreviations*:** PPDK, pyruvate phosphate dikinase; *Bm*, *B. malayi*; *w*Bm, *B. malayi Wolbachia*

***Running Title*:** *Wolbachia* pyruvate phosphate dikinase

Résumé

L'etude genomique des voies glycolytique et néoglucogénique du symbiote *Wolbachia* de *Brugia malayi* (wBm) a révélé que *w*Bm ne possède pas la pyruvate kinase. *w*Bm pourrait à la place utiliser l'enzyme pyruvate phosphate dikinase (PPDK ; ATP :pyruvate, orthophosphate phosphotransferase, EC 2.7.9.1). PPDK catalyse la conversion réversible de l'AMP, du PPi et du phosphoenolpyruvate en ATP, Pi et pyruvate. La plupart des organismes, y compris les mammifères possèdent exclusivement la PK. Par conséquent, l'absence de PPDK chez les mammifères rend cette enzyme attractive en tant que cible thérapeutique. Dans cette étude, nous avons cloné et exprimé une *w*Bm-PPDK active, fournissant ainsi un aperçu du métabolisme énergétique du symbiote. Nos résultats supportent le développement de *w*Bm-PPDK en tant que cible thérapeutique prometteuse au sein d'une approche anti-symbiote dans le contrôle des filarioses.

Mot-clés: *Wolbachia, Brugia malayi*, glycolyse, néoglucogenèse, pyruvate phosphate dikinase

Abstract

Genome analysis of the glycolytic/gluconeogenic pathway in the *Wolbachia* endosymbiont from the filarial parasite *Brugia malayi* (*w*Bm) has revealed that *w*Bm lacks pyruvate kinase (PK) and may instead utilize the enzyme pyruvate phosphate dikinase (PPDK; ATP:pyruvate, orthophosphate phosphotransferase, EC 2.7.9.1). PPDK catalyses the reversible conversion of AMP, PPi and phosphoenolpyruvate into ATP, Pi and pyruvate. Most organisms, including mammals possess exclusively PK. Therefore the absence of PPDK in mammals makes the enzyme an attractive *Wolbachia* drug target. In the present study, we have cloned and expressed an active *w*Bm-PPDK, thereby providing insight into the energy metabolism of the endosymbiont. Our results support the development of *w*Bm-PPDK as a promising new drug target in an anti-symbiotic approach to controlling filarial infection.

Keywords: *Wolbachia, Brugia malayi*, glycolysis, gluconeogenesis, pyruvate phosphate dikinase

Chapter 4

Wolbachia are intracellular alphaproteobacteria found in arthropod and nematode hosts. It is estimated that 20-80% of arthropod species are infected (Jeyaprakash and Hoy, 2000; Werren and Windsor, 2000), whereas in nematodes, *Wolbachia* have exclusively been found in vector-transmitted Onchocercidae such as the human parasites *Brugia malayi*, *Onchocerca volvulus*, *Wuchereria bancrofti* and *Mansonella ozzardi* (Casiraghi *et al.*, 2001; Casiraghi *et al.*, 2005), and the animal filarial parasite *Dirofilaria immitis* (Sironi *et al.*, 1995). The presence of *Wolbachia* in oocytes, developing eggs and microfilaria (McLaren *et al.*, 1975; Kozek and Marroquin, 1977) indicate that the bacteria are maintained in the population by vertical transmission. Various studies have shown that nematode *Wolbachia* are required for embryogenesis, larval development and adult worm survival (Pfarr and Hoerauf, 2007). This has indicated the use of antibiotics as an alternative approach to the treatment and control of filarial parasites. However, the long treatment regimens that are required using doxycycline in humans present logistical problems for mass drug administration and compliance, necessitating the need to develop improved methods that target *Wolbachia*.

The sequenced genome of the *Wolbachia* endosymbiont from *B. malayi* (*w*Bm) (Foster *et al.*, 2005) offers an unprecedented opportunity to identify new *Wolbachia* drug targets. Genome analysis of the glycolytic/gluconeogenic pathway has revealed that *w*Bm lacks pyruvate kinase (PK) and may instead utilize the enzyme pyruvate phosphate dikinase (PPDK; ATP:pyruvate, orthophosphate phosphotransferase, EC 2.7.9.1). PPDK is a ~96 kDa protein which functions as a dimer (e.g *Trypanosoma brucei* (Cosenza *et al.*, 2002)) or a tetramer (e.g. *Entamoeba histolytica* (Saavedra-Lira *et al.*, 1998)), and catalyses the reversible conversion of AMP, PPi and phosphoenolpyruvate into ATP, Pi and pyruvate (Wood *et al.*, 1977). PPDK is an energy conserving, reversible alternative to PK producing four ATP molecules per glucose molecule compared with the two produced by PK in standard glycolysis (Mertens, 1993). Most organisms, including mammals possess exclusively PK. Therefore the absence of PPDK in mammals makes the enzyme an attractive *Wolbachia* drug target. Since there is no protein sequence

homology between PPDK and PK, it is feasible that highly specific inhibitors of PPDK activity may be identified.

The goals of the present study were to clone and express the predicted PPDK from *w*Bm (*w*Bm-PPDK) to gain insight into the energy metabolism of this endosymbiont and to develop the enzyme as a drug target.

Specific primers were designed for PCR amplification of *wBm-PPDK* from *B. malayi* genomic DNA. The forward primer was designed so that the PCR product would exclude the first 48 nucleotides of the predicted open reading frame (GenBank accession number AE017321) and the recombinant *w*Bm-PPDK would, therefore, begin with the methionine at residue 17 of the deduced protein. This strategy was adopted since this methionine residue aligned with the initiation methionine of known PPDK proteins and proteins translated from various genome sequences. The forward primer *w*Bm-PPDK-BamHI (5'- GATTCAGGATCCATGGAAGAAAAGTTAATATACTACT-3') contained a BamHI site (underlined) and the reverse primer *w*Bm-PPDK-XhoI (5'-GTAGTACTCGAGTCCAACACACTTAGATTTCTGA-3') contained an XhoI site (underlined). Reactions were carried out using 0.5 μM of each primer, 200 μM of each deoxynucleotide, 1 Unit of Vent DNA Polymerase (New England Biolabs), 1X ThermoPol Reaction Buffer and the following conditions: 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 3 min, and 1 cycle at 72°C for 10 min. Reaction product corresponding to the predicted full length ~2.6 kb insert was purified and digested using BamHI and XhoI, and ligated into pET21a(+) vector (Novagen) digested by the same pair of restriction enzymes. Plasmid DNA was isolated, and the insert verified by sequencing. The *wBm-PPDK* coding frame is 2622 bp in length, and the translated protein (873 aa) has a predicted molecular mass of 96.4 kDa with a pI of 6.2. The p*I* of *w*Bm-PPDK is lower than that of the *T. brucei* PPDK (pI 8.25) but more consistent with the enzymes characterized from other sources. The higher pI of the protozoan enzyme is likely due to its glycosomal location (Bringaud *et al.*, 1998).

Phylogenetic (Figure 1A) and sequence (Figure 1B) analyses indicate that *w*Bm-PPDK is an ortholog of the previously described PPDK enzymes from the protozoan

parasites *T. brucei, E. histolytica* and *Giardia duodenalis,* and the more closely related bacteria *Clostridium symbiosum* and *Anaplasma marginale.* Interestingly, *w*Bm-PPDK shares a comparable level of identity (47-57%) with each of these organisms, and the levels do not increase significantly when the parasite (51-53%) or other bacterial enzymes (49%) are compared to each other.

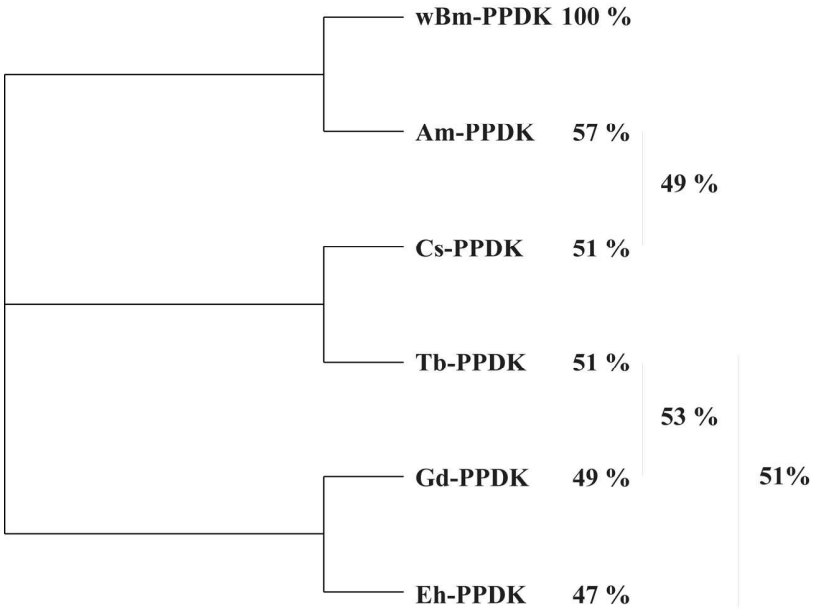

Figure 1A: Cladogram of predicted full-length PPDK sequences from various organisms.
The percentage of amino acid sequence identity of PPDKs from *Anaplasma marginale* (Am-PPDK), *Clostridium symbiosum* (Cs-PPDK), *Trypanosoma brucei* (Tb-PPDK), and *Entamoeba histolytica* (Eh-PPDK) to *wB. malayi* PPDK (*w*Bm-PPDK) is shown. The relatedness of the organisms within the protozoan parasites and bacterial groups is also indicated.

Such sequence similarities suggest that the enzymes are evolutionarily related and that they have a conserved three-dimensional structure. *w*Bm-PPDK contains the highly conserved catalytic histidine and the other 10 residues (Figure 1B) predicted to be involved in catalysis, based on structural and biochemical studies on *C. symbiosum* PPDK (McGuire *et al.*, 1996; McGuire *et al.*, 1998; Ye *et al.*, 2001; Herzberg *et al.*, 2002).

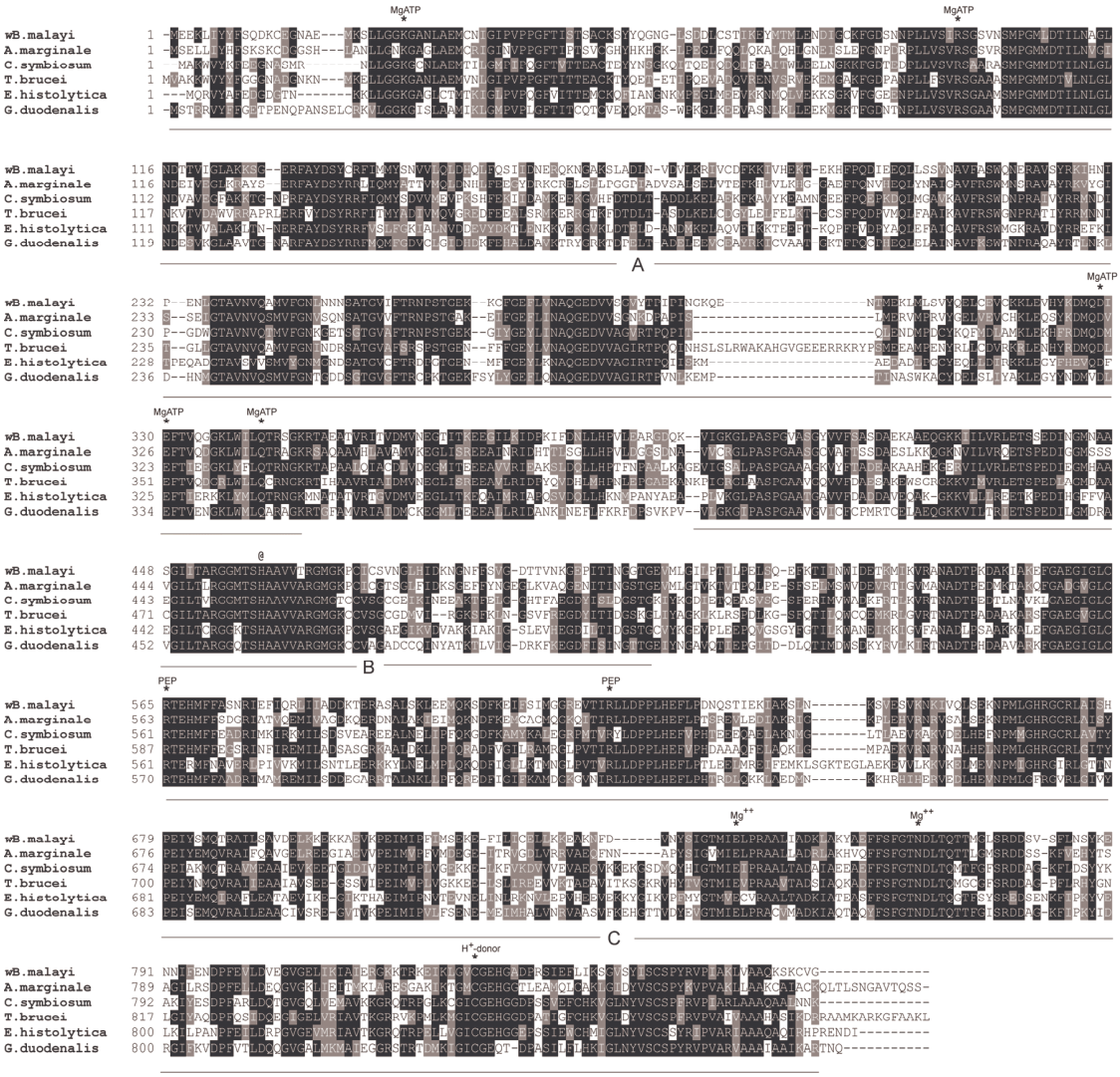

Figure 1B: Alignment of the deduced amino acid sequences of various PPDK enzymes.

The amino acid sequence of PPDK enzymes from the *Wolbachia* endosymbiont of *Brugia malayi* (*wB. malayi,* GenBank AE017321) is aligned with the enzyme from the closely related bacterium *Anaplasma marginale* (Genbank CP000030), active recombinant PPDKs from *Trypanosoma brucei* (Genbank AF048689), *Entamoeba histolytica* (Genbank XM_652240), *Giardia duodenalis* (Genbank Z54168), and the structurally characterized *Clostridium symbiosum PPDK* (Genbank P22983). Residues that are identical in at least 50% of the sequences are shaded in black, and the conserved amino acids are in grey. The 3 domains of the enzymes (indicated A-C), the catalytic histidine (@) and 10 other amino acids (asterisk) involved in catalysis are indicated (Ye *et al.*, 2001; Herzberg *et al.*, 2002). The residues involved in the binding of ATP, magnesium (Mg), phosphoenolpyruvate (PEP), and the cysteine hydrogen (H+) donor are indicated. The alignment was performed with ClustalW and displayed with BOXSHADE (www.ch.embnet.org/software/BOX_form.html).

The reaction (reversible) catalysed by PPDK occurs in three steps (Xu *et al.*, 1995), where the outcome depends on the organism (glycolysis and ATP formation, or phosphoenolpyruvate synthesis):

E-His + ATP + Pi ↔ E-His-PP·AMP·Pi

E-His-PP·AMP·Pi ↔ E-His-P + AMP + PPi

E-His-P + Pyruvate ↔ E-His + PEP

The enzyme is composed of three domains (indicated in figure 1B), each with an important role. The central domain is small and spherical and its catalytic histidine residue is responsible for the transport of the phosphate group between the active sites of the two terminal domains, while the N-terminal domain (containing the ATP/Pi active site) catalyzes steps 1 and 2 and the C-terminal domain (containing the pyruvate active site) performs the third step of the reaction (Ye *et al.*, 2001; Herzberg *et al.*, 2002). It has been speculated that the enzyme might have evolved through the process of gene duplication and fusion leading to the linkage of three proteins whose structures coevolved to support a novel function (Lin *et al.*, 2006).

*w*Bm-PPDK was expressed with a C-terminal His-tag in T7 Express Competent *Escherichia coli* (New England Biolabs), with and without the RIL plasmid encoding the rare tRNAs for arginine, isoleucine and leucine. A number of experiments were performed to maximize expression, solubility and yield. These included varying growth temperature, timing and length of induction with varying amount of isopropyl •-D-thiogalactopyranoside (IPTG, American Bioanalytical). Optimum conditions for production of soluble recombinant *w*Bm-PPDK involved growth at 37°C in *E. coli* containing the RIL plasmid and induction with 0.1 mM IPTG overnight at 15°C. The His-tagged protein was extracted and purified on nickel resin (Qiagen) under native conditions according to the manufacturer's instructions with some modifications in the buffer composition. In particular, 20 mM •-mercaptoethanol and 10% glycerol were added to maintain solubility and activity. An elution buffer (40 mM NaH_2PO_4, 300 mM NaCl, 20 mM •-mercaptoethanol, 10% glycerol, pH 8) containing varying amounts of imidazole was evaluated for optimal release of the His-tagged protein from the nickel

resin and 50 mM was found sufficient to release the majority of the protein in a purified form. Purity of the protein was estimated by SDS-PAGE and the protein concentration determined with the Bradford assay. The apparent molecular weight (~96 kDa) on SDS-PAGE was consistent with the predicted molecular weight (figure 2A). Fractions were tested for activity, pooled, and dialyzed overnight at 4°C against a storage buffer (100 mM Tris pH 6.5, 300 mM NaCl, 1 mM EDTA, 20 mM •-mercaptoethanol, 5 mM $MgCl_2$, 20% glycerol). The dialyzed solution was then aliquoted, flash frozen and stored at -80°C.

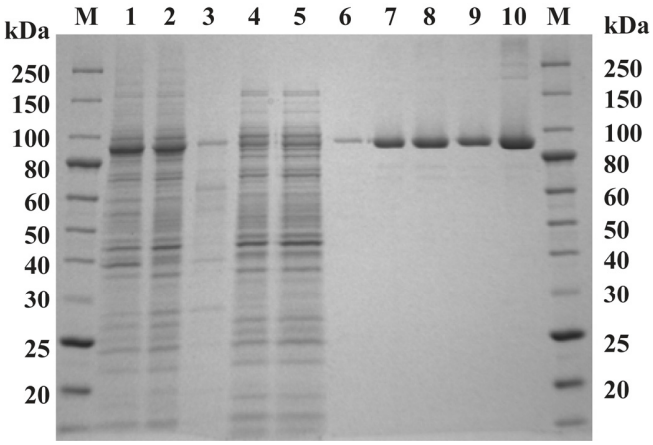

Figure 2A: Purification of recombinant *w*Bm-PPDK.

M: Protein Ladder (NEB P7703); *lane 1*: *E. coli* total extract after sonication; *lane 2*: soluble *E. coli* proteins; *lane 3*: insoluble *E. coli* proteins; *lane 4*: flow through from the Ni-column; *lanes 5 and 6*: column washes; *lanes 7-10*: consecutive fractions eluted with 50 mM imidazole.

PPDK activity was determined in the forward (glycolytic) direction (phosphoenolpyruvate to pyruvate) using a spectrophotometric assay slightly modified from a previously published method (South and Reeves, 1975) that measures enzyme activity indirectly by monitoring the oxidation of NADH to NAD at 340 nm. Reactions were performed at room temperature for 5 min with data collected at 10 sec intervals using a Beckman DU 640 spectrophotometer. PPDK was added to 1 ml assay buffer (50 mM imidazole pH 6.3, 20 mM NH_4Cl, 10 mM $MgCl_2$, 0.15 mM NADH, 1 mM phosphoenolpyruvate, 0.3 mM AMP, 2 mM PPi, 3U L-lactic dehydrogenase). Control assays were performed to provide a baseline (without PPDK) and to ensure the function of the coupling enzyme (using sodium pyruvate). A specific activity of 0.62 units/mg (Figure 2B) was determined where one unit is defined as the conversion of 1.0 μmole NADH to NAD per minute. This level of activity is less than the values reported for recombinant enzymes from *T. brucei* (2.5 units/mg, (Bringaud *et al.*, 1998)), *G.*

duodenalis (10-17 units/mg, (Hiltpold *et al.*, 1999)) and *E. histolytica* (10-13 units/mg, (Saavedra-Lira *et al.*, 1998)). However, like *T. brucei* PPDK and other PPDK enzymes (Evans and Wood, 1971; Milner *et al.*, 1975; Cosenza *et al.*, 2002), *w*Bm-PPDK is relatively unstable (data not shown).

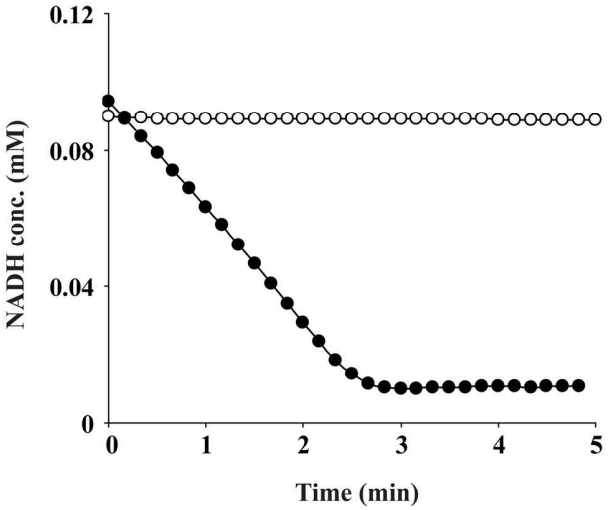

Figure 2B: Activity of recombinant *w*Bm-PPDK.

Recombinant *w*Bm-PPDK (●). A control without enzyme (○) was included.

Conversion of phosphoenolpyruvate to pyruvate is indicated by a decrease in NADH concentration as measured by its absorbance at 340 nm. The consumption of NADH is directly proportional to PPDK activity.

The absence of PPDK from mammals and its important role in the parasites *E. histolytica* and *G. lamblia* (Saavedra-Lira and Perez-Montfort, 1996; Feng *et al.*, 2008) has stimulated considerable interest in the enzyme as a potential target for the design of novel anti-protozoan drugs. PPDK is considered particularly important in trypanosomes since it appears to provide a link between breakdown of sugars and fatty acids via glycolysis and fatty acid β-oxidation for other biosynthetic pathways (Acosta *et al.*, 2004). *T. brucei* PPDK is the most characterized of the protozoan enzymes (Bringaud *et al.*, 1998) and a 3.0 Å resolution crystal structure is available (Cosenza *et al.*, 2002) which shows considerable similarity to the structure of the bacterial (*C. symbiosum*) enzyme (Herzberg *et al.*, 1996). The similarity between *w*Bm-PPDK and the previously characterized PPDK enzymes makes it tantalizing to speculate that a single inhibitor may be effective. Imidodiphosphate and oxalate have been shown to inhibit the activity of *Giardia* PPDK (Hiltpold *et al.*, 1999), and molecular modeling studies are underway on *E. histolytica* PPDK to enable the *in silico* screening for novel inhibitors (Stephen *et al.*, 2007). Interestingly, PPDK is also being targeted as a means to control weed species of plants and a rapid throughput screening assay for inhibitors has been developed (Doyle *et*

al., 2005). Targeting *w*Bm-PPDK appears particularly promising since the *Wolbachia* genome does not predict any other enzyme capable of interconverting phosphoenolpyruvate and pyruvate. The genomes of the closely related rickettsial organisms, *Anaplasma* (anaplasmosis) and *Ehrlichia* (ehrlichiosis and heartwater disease) as well as those of *Porphyromonas gingivalis* (periodontal disease) and *Treponema pallidum* (syphilis) also predict only PPDK for this metabolic conversion. Significantly, in the case of the rickettsial organisms, *Wolbachia*, *Anaplasma* and *Ehrlichia,* there is also a lack of any phosphoenolpyruvate carboxylase or phosphenolpyruvate carboxykinase that interconvert oxaloacetate and phosphoenolpyruvate in many organisms. Therefore, in these rickettsial organisms, PPDK appears to be essential for any form of glycolysis or gluconeogenesis.

In *E. histolytica* and *G. duodenalis*, the precise physiological role of PPDK is still under discussion (Hiltpold *et al.*, 1999; Varela-Gomez *et al.*, 2004). Further studies are needed to elucidate the exact role of *w*Bm-PPDK and the direction of the catalyzed reaction (glycolysis versus gluconeogenic), and to determine if the enzyme contributes to the metabolic activity of filarial parasites.

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Chapter 5: Identification of peptides ligands of independent phosphoglycerate mutase using phage display librairies

Chapter 5

Identification de peptides se liant à la phosphoglycérate mutase indépendante par utilisation de bibliothèques d'exposition sur phages

La présentation de peptides à séquences aléatoires à la surface de phages, et le lien direct avec la séquence nucléotidique fournit un outil très utile pour rapidement et efficacement sélectionner des peptides se liant à une cible. Cette approche a été utilisée avec succès pour identifier plusieurs inhibiteurs d'enzymes. Afin de développer les PGMis en tant que potentielle cible thérapeutique chez les nématodes, *Wolbachia* et les trypanosomes, nous avons décidé d'utiliser cette technique pour chercher des peptides inhibiteurs de l'enzyme. Dans cette étude, j'ai criblé trois bibliothèques de phages exprimant des peptides aléatoires de 7 (linéaires ou cycliques) ou 12 acides aminés afin d'identifier des peptides se liant à la phosphoglycérate mutase indépendante d'*O. volvulus* purifiée. Nous avons décidé de réaliser le criblage en solution plutôt que sur support solide (méthode conventionnelle) afin de préserver la conformation correcte de l'enzyme et d'éviter de sélectionner des peptides qui pourraient se lier au support solide plastique. Un tour de sélection négatif a également été utilisé pour éliminer les peptides non spécifiques réagissant avec les billes ou l'étiquette utilisée. J'ai utilisé deux conditions d'élution différentes, l'une favorisant la sélection de peptides se liant au site actif de l'enzyme et l'autre basée sur une élution non spécifique en conditions dénaturantes. Apres trois tours de sélection, plusieurs séquences de peptides consensus ont été obtenues. Ceci a été problématique, car une seule séquence consensus n'a pas été obtenue. Malgré cela, certains motifs ont été identifiés et plusieurs peptides ont montrés avoir une affinité satisfaisantes pour la PGMi en test ELISA. Malheureusement ces peptides ne possèdent pas d'activité inhibitrice sur l'enzyme dans les conditions testées. De plus amples études sont requises pour identifier des peptides se liant avec grande affinité au site actif de l'enzyme.

Identification of peptide ligands of independent phosphoglycerate mutase using phage display libraries

The display of a randomized peptide library at the surface of phage, and the direct link with the nucleotide sequence provides a very useful tool to quickly and efficiently select and identify peptides which bind a target. This approach has been used successfully to identify various enzyme inhibitors. In order to further develop iPGM as a potential drug target in nematodes, *Wolbachia* and trypanosomes, we decided to use this technique to search for peptide inhibitors of the enzyme. In this study, I screened three phage display libraries displaying randomized 7-mer (linear or cyclic) or 12-mer peptides to identify peptides which bind purified recombinant *Onchocerca volvulus* independent phosphoglycerate mutase. We decided to perform the panning in solution rather than on a solid surface (conventional method) to preserve the correct confirmation of the enzyme and to avoid selection of peptides that may bind to the solid (plastic) support. A negative round of selection was also used to remove non-specific peptides reacting with the beads, or the tag used. I used two different elution conditions, one favoring selection of peptides binding the active site of the enzyme and the other based on non-specific elution under denaturing conditions. After three rounds of panning the peptide sequences were analyzed. This was challenging since a single consensus sequence was not obtained. Nevertheless, some shared motifs were identified and several peptides were then shown to have reasonable affinity for iPGM in phage ELISA. Unfortunately these peptides did not possess enzyme inhibitory activity under the conditions used. Further studies are needed to identify peptides with high affinity binding to the active site of the enzyme.

Chapter 5

Identification of peptide ligands of independent phosphoglycerate mutase using phage display libraries

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Abbreviations: iPGM, independent phosphoglycerate mutase; *Ov*, *Onchocerca volvulus*

Résumé

Les maladies parasitaires sont un lourd fardeau pour les pays en voie de développement et l'apparition de résistance nécessite la recherche de nouvelles cibles thérapeutiques et de nouveaux composés leader. La phosphoglycérate mutase indépendante (PGMi) a été identifiée comme potentielle cible thérapeutique chez les filaires dont la filaire humaine *Onchocerca volvulus*. Les phosphoglycérates mutases (PGM, EC 5.4.2.1) catalysent l'isomérisation du 3-phosphoglycérate et du 2-phosphoglycérate dans la glycolyse et la néoglucogenèse. Dans cette étude, trois librairies d'exposition sur phage exprimant des peptides de 7 (linéaires ou circulaires) ou 12 acides aminés de séquences aléatoires ont été criblées pour identifier des peptides s'attachant à la PGMi d'*O. volvulus*. La sélection a été réalisée en solution plutôt que sur une surface solide pour préserver la conformation de l'enzyme et pour éviter la sélection de peptides qui pourrait s'accrocher à la surface solide. Deux types de PGM différentes existent dans la nature, un type qui requiert le 2,3-bisphosphoglycérate en tant que cofacteur (PGMd) et un autre type qui ne le requiert pas (PGMi). Un tour de sélection négative a également été utilisé pour éliminer les peptides non spécifiques réagissant avec les billes ou l'étiquette utilisée. Deux conditions d'élution différentes ont été appliquées, l'une favorisant la sélection de peptides s'accrochant au site actif, l'autre basée sur une élution non spécifique dans des conditions dénaturantes. Apres trois tours de sélection, plusieurs séquences de peptides consensus ont été obtenues. Plusieurs peptides ont ensuite montrés avoir une affinité acceptable pour la PGMi d'*O. volvulus* en test ELISA.

Mots-clés : Phage display, phosphoglycérate mutase indépendante, *Onchocerca volvulus*, Inhibiteurs.

Abstract

Parasitic diseases are a heavy burden for developing countries and the appearance of drug resistance necessitates research on new drug targets and the identification of new lead compounds. Independent phosphoglycerate mutase (iPGM) has been identified as a potential drug target in parasitic nematodes including the human filarial parasite *Onchocerca volvulus*. Phosphoglycerate mutase (PGM, EC 5.4.2.1) catalyzes the isomerization of 3-phosphoglycerate and 2-phosphoglycerate in glycolysis and gluconeogenesis. In this study, three phage display libraries displaying randomized 7-mer (linear or cyclic) or 12-mer peptides were screened to identify peptides which bind *O. volvulus* iPGM. Panning was performed in solution rather than on a solid surface to preserve the correct confirmation of the enzyme and to avoid selection of peptides that may bind to the solid support. A negative round of selection was also used to remove non-specific peptides reacting with the beads, or the tag used. Two different elution conditions were used, one favoring selection of peptides binding the active site of the enzyme and the other based on non-specific elution under denaturing conditions. After three rounds of panning various consensus peptide sequences were obtained. Several peptides were then shown to have reasonable affinity for *O. volvulus* iPGM in phage ELISA.

Keywords: Phage display, independent phosphoglycerate mutase, *Onchocerca volvulus*, inhibitor.

Introduction

Parasites cause a number of important diseases in humans, animals and plants. They are found worldwide, however they are a particular problem in developing countries. It is estimated that 150 million people in the world are infected with a group of nematodes called filarial nematodes and more than a billion people are at risk (WHO, 2003). Control of filariasis currently relies on the drugs ivermectin, albendazole and diethylcarbamazine. Despite, their relative efficacy on immature worms, these drugs are considered inadequate because of limited potency on adult worms, side effects or toxicity. In the case of ivermectin, increasing use worldwide has resulted in concerns for the development of drug resistance. Therefore there is an urgent need for new and improved drugs.

Target-based drug discovery represents one approach to identifying new therapeutics. The recently completed genome sequence of the human filarial parasite *Brugia malayi* (Ghedin *et al.*, 2007) has enabled a genome-wide search for new nematode drug targets. A bioinformatic method was developed that relies on functional information available from the related free-living nematode *Caenorhabditis elegans*, and uses a number of factors to rank the targets including their absence from humans (Foster *et al.*, 2005; Kumar *et al.*, 2007). One particularly interesting target identified using this approach is the enzyme co-factor independent phosphoglycerate mutase (iPGM). PGMs catalyze the interconversion of 2- and 3-phosphoglycerate (2-PG and 3-PG) in the glycolytic and gluconeogenic pathways. Although these pathways are highly conserved among different organisms, two distinct PGM enzymes are known to exist, iPGM and the cofactor dependent phosphoglycerate mutase, dPGM. Mammals, in addition to some bacteria and fungi, possess exclusively dPGM whereas other organisms may have exclusively iPGM, or both forms (Carreras *et al.*, 1982; Fothergill-Gilmore and Watson, 1990; Fraser *et al.*, 1999). There is no protein sequence or structure homology between the two forms and they operate via different biochemical mechanisms (Jedrzejas, 2000; Rigden *et al.*, 2002). Previous studies have shown that parasitic and free-living nematodes possess only the iPGM form (Zhang *et al.*, 2004). The high degree of

conservation among nematode iPGM enzymes and the fact that the enzyme has an essential role in *Caenorhabidits elegans* development indicates that iPGM likely represents a broad-spectrum target in parasitic nematodes (Zhang *et al.*, 2004). To date, there have been no inhibitors of iPGM documented. Vanadate inhibits the enzyme activity of dPGM but not iPGM (Carreras *et al.*, 1980), and more recently a dPGM inhibitory synthetic peptide has been described which inhibits glycolysis in tumor cells resulting in cell growth arrest (Engel *et al.*, 2004).

Random peptide libraries displayed on the surface of filamentous bacteriophage, each of which expresses a unique peptide sequence on its surface, are widely used as tools for the discovery of ligands for antibodies, cell surface receptors and enzymes. This approach has also proved useful for the identification of various enzyme inhibitors (Hyde-DeRuyscher *et al.*, 2000; El Zoeiby *et al.*, 2003; Lunder *et al.*, 2005; Sanschagrin and Levesque, 2005). Phage display links an affinity-selectable function (the displayed peptide) to the DNA encoding that function, allowing selection of individual binding clones by iterative cycles of *in vitro* panning and *in vivo* amplification (Smith and Petrenko, 1997; Barbas *et al.*, 2001). This permits easy screening of a large number of sequences in a short time.

In this report we describe the screening of three different phage display libraries for the identification of peptides which bind nematode iPGM. Recombinant iPGM from the human filarial parasite *Onchocerca volvulus* was used as the target molecule and panning was performed in solution rather than on a solid surface to preserve the correct conformation of the enzyme. Two different elution conditions were used, one designed to favor the selection of inhibitory peptides binding to the active site of the enzyme and the other based on non-specific elution under denaturing conditions.

Materials and Methods

The Ph.D.-C7C^{TM}, Ph.D.-12^{TM} and Ph.D.-7^{TM} libraries (New England Biolabs) were used to identify peptides that bind to *Onchocerca volvulus* independent phosphoglycerate mutase (Ov-iPGM). Purified, active recombinant Ov-iPGM was

produced with both a T7-tag at the N-terminus and a His-tag at the C-terminus as previously described (Raverdy *et al.*, 2007). The libraries consist of randomized 12- and 7-mers (cyclic in the case of the disulfide-constrained C7C) fused to the N-terminus of a minor coat protein (pIII) of M13 phage. This protein is responsible for attachment of the phage to bacteria and resulting infection.

Panning phage display libraries

Panning against the target (100 nM) was performed for 20 min at room temperature (RT) in a final volume of 200 µl TBST (1X TBS + 0.1% (v/v) Tween-20) containing $2X10^{11}$ plaque-forming units (pfu). The Ph.D.-12™ and Ph.D.-7™ libraries were combined prior to screening, whereas the Ph.D.-C7C™ was screened independently. 100 nM of T7-tag antibody was then added and the solution was incubated for 5 min at RT. The phage-target mix was then transferred to a tube containing 50 µl of a 50% aqueous suspension of protein A magnetic beads (NEB, USA), previously blocked (1h at 4°C with occasional mixing in 1 ml buffer containing 5mg/ml BSA in 0.1 M $NaHCO_3$, pH 8.6, 0.02% NaN_3) and washed (4 times with 1 ml TBST), and incubated at RT for 15 min after gentle mixing. The beads were pelleted and washed 10 times with 1 ml TBST. Bound phage was eluted by resuspending the beads in either 1 ml 1 mM 3-phosphoglycerate and incubating overnight at RT, or with 1 ml 0.2 M glycine-HCl (pH 2.2) for 10 min at RT. The latter eluate was immediately neutralized with 150 µl 1 M Tris-HCl, pH 9.1. For the Ph.D.-C7C™ library, 1 mM 3-PG or 5 mM DTT was used for elution. A volume of 10 µl of each eluate was titrated as described below.

In order to increase the specificity of the selection, several rounds (usually 3) of panning were performed. For the second and third round of panning, the amplified phage input remained at $2x10^{11}$ pfu and the Tween-20 concentration of the wash buffer was increased to 0.5%. Phage(s) binding nonspecifically to the antibody or protein-A beads were removed in a negative selection step after the first round of panning and amplification. This involved incubating amplified phage at a concentration of $2x10^{11}$ pfu with 100 nM of the T7 Tag antibody for 5 min at RT. Beads (blocked and washed) were

added and incubated 20 min at RT. The supernatant was then used for the second round of panning.

Amplification

Eluates were amplified by addition to a 20 ml culture of ER2738, grown to early log, and further incubation at 37°C for 4.5 hours with vigorous shaking. The culture was centrifuged at 13,800 x g for 10 min at 4°C. The supernatant was collected and the phage precipitated at 4°C overnight by addition of 1/6 volume of 20% PEG/2.5M NaCl. The phage were pelleted at 13,800 x g for 15 min at 4°C, and resuspended in 1 ml TBS. Following centrifugation for 5 min at 4°C to remove residual cells, a second precipitation was performed by adding 1/6 volume 20% PEG/2.5 M NaCl and incubating on ice for 15-60 min. The solution was then microcentrifuged at maximum speed for 10 min at 4°C. The final pellet was resuspended in 200 µl TBS, 0.02% NaN_3 and the titer of the resulting phage stock was determined.

Titration

The phage concentration was determined by serial dilution of the phage in LB medium and infection of 200 µl of a mid log ER2738 culture, followed by plating of each dilution on the X-gal/IPTG medium to enable rapid identification of the correct plaques by their blue color. Blue plaques were counted after overnight incubation.

Sequencing

Single-stranded DNA sequencing was performed to determine if the panning was converging on particular sequences. After the third round, un-amplified phage were diluted to yield 10-100 plaques per plate. Individual plaques were then selected and cultured independently. Phage in the supernatant (500 µl) were precipitated at RT for 10 min by adding 20% PEG/2.5 M NaCl (200 µl). Following centrifugation, the pellet was resuspended in 100 µl iodide buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, 4 M NaI) to denature coat proteins. DNA was precipitated at RT for 10 min by adding 250 µl 95% ethanol. The sample was then microcentrifuged for 10 min at maximum speed, and the

pellet washed in 70% ethanol and dried under vacuum. The DNA was resuspended in 30 µl TE (10 mM Tris-HCl (pH 8.0), 1 mM EDTA) and used as a template for dideoxy DNA sequencing using the -96 M13 sequencing primer (5′-CCCTCATAGTTAGCGTAACG- 3′, NEB).

Sequences were analyzed and all non-gapped pairwise alignments with a minimum overlap of 7 residues were generated. Patterns consisting of identical aligned residues (possibly separated by non-identical residues) having a minimum number of identical residues were extracted. When multiple patterns were present in an alignment, the highest scoring pattern was chosen. After the all versus all pattern generation step, identical patterns were merged and the sequences containing the patterns were pooled. The frequency of patterns was taken in account for the analysis.

Phage ELISA

Microtiter plate wells were coated with 200 µl Ov-iPGM (10 µg/ml) in 0.1 M $NaHCO_3$ (pH 8.6) overnight at 4°C and blocked with 200 µl blocking buffer (5mg/ml BSA in 0.1 M $NaHCO_3$, pH 8.6, 0.02% NaN_3) at RT for 1h. The phage clones at concentrations of 1.8-3.0 x 10^{11} pfu/µl were diluted in 200 µl wash buffer (0.5% Tween-20) and added to the micro-plate and incubated 1h at RT. The plate was then washed 6 times with wash buffer. Horseradish peroxidase-labeled mouse anti-M13 antibody (1:2500) in blocking buffer was added and incubated for 1h at RT. Following washing, 200 µl of substrate solution (0.22 mg/ml, diammonium 2,2'-azino-di-(3-ethylbenzthiazoline sulfonate in 50 mM citric acid and 1.7 µl 30% H_20_2/ml, pH 4.0) was added and the plates were incubated for 60 min at RT. Absorbance at 405 nm was then determined.

Inhibition of enzyme activity

Peptides corresponding to phage clones that showed affinity for the target were synthesized and evaluated for their ability to inhibit the enzyme activity of Ov-iPGM. PGM activity was measured by measuring the consumption of NADH, which is monitored at 340 nm as described (Zhang *et al.*, 2004; Raverdy *et al.*, 2007). Inhibition

was determined following pre-incubation (1h) of the enzyme (1 μg) with various amount of peptides diluted in 1 M Tris buffer (pH 7.4).

Results and Discussion

The availability of genome sequence from infectious disease organisms has resulted in a rapidly expanding list of potential new drug targets and a need for a facile, readily available method for the discovery of new inhibitors. Phage display has been used effectively to screen large collections of random sequence peptides for variants which bind a target protein (Smith and Petrenko, 1997). Some of these sequences may also possess enzyme inhibitory properties (Hyde-DeRuyscher *et al.*, 2000; El Zoeiby *et al.*, 2003; Lunder *et al.*, 2005; Sanschagrin and Levesque, 2005).

We used three M13 phage display libraries, displaying randomized 7-mer (linear or cyclic) or 12-mer peptides, to search for peptide sequences which bind to the enzyme target iPGM. Highly purified, active recombinant enzyme was used for the study (Raverdy *et al.*, 2007). Assurance of the quality of the target protein, and in particular purity, is one of the most important preconditions for successful selection of ligands from phage display libraries (Lunder *et al.*, 2005). The conditions used to elute the bound phage can also profoundly influence the diversity and affinity of the peptides selected (Lunder *et al.*, 2005). Phage that bound to Ov-iPGM were released using two distinct methods, either with a low pH or DTT buffer that elutes all phage including high-affinity clones, or with the enzyme substrate 3-phosphoglycerate to favor the selection of inhibitory peptides binding to the active site of the enzyme. Twenty randomly picked phage clones from each type of elution were sequenced after three rounds of panning with either the combined Ph.D.-12^{TM} and Ph.D.-7^{TM} libraries, or the Ph.D.-C7C^{TM} library **(Table 1).**

Ph.D7/Ph.D12 librairies		Ph.DC7C library	
Low pH elution	Substrate elution	DTT elution	Substrate elution
LMTPNQTQRNRL	* QMFEGPMSRLHL	CSMTPVQQC	CSTTPVQQC
	QMFEGPMSRLHL	* CATWAPQQC	CNTSAPQQC
	QMFEGPMSRLHL	CRPVAPQQC	CQTMANQQC
	QMFEGPMSRLHL	CRTTTPQQC	
		CNFARPQQC	
* NYSHLRVKLPTP	NYSHLRVKLPTP	CNSSSPQQC	
QYSHIGNYAPQP	NYSHLRVKLPTP		
SYSPLTIVNPKV	NYSHLRVKLPTP	CTPLGPLWC	CTPLGPLQC
SYLQSKSYFLPP	VYSPRWWQLNIS	* CTPYFPQQC	CTPFSPQWC
		CEPSGPQQC	CTPSAPQLC
* NFAAIAPQQFFL	* DAPMTGRQMSTN	CDPIAPQMC	CLPTGPQQC
NFAAIAPQQFFL	DAPMTGRQMSTN	CSSFAPQMC	CESAGWQQC
NFAAIAPQQFFL			
NFAAIAPQQFFL	* SEPMHRPMSDMQ	CPSMSPKQC	* CPSMGPQTC
	SEPMHRPMSDMQ	CSSMSKQQC	CMQMGPQIC
* QSTYENPMSPQM		CPAMSHQQC	
QSTYENPMSPQM	MMGPQPNLLRDQ	CKSMSYQQC	
QSTYENPMSPQM	NTWPMHRPLLNQ		
THTSMTPWPSGM		CTPYSPQTC	* CAPYSAQLC
	FGPNFSHLYPRP		
THYGVPYMPRVL	WSASYLIPNQGW	* CTQPMSRLC	CTRPTSILC
THYGVPQMPRVL	* QMTPPMQ	CWTPFSRLC	
	SAGWGGH	CYGPPTRLC	
QIHRTMTPFQLI		* CYSPLSQQC	CYSPHNQTC
QPFALMTGNQQS			
			* CVSPMRPLC
* APMSLRHMKQSA			CQSPMSKLC
GPTSHLQMYNSG			
			* CMSPKQQWC
TLLKPMSSNWRM			CMSAPWGHC
			CMTPRQLMC
			CILTPHAQC
			CSYSAYSQC

Table 1: Selection of iPGM-binding peptides from various phage display libraries using different elution protocols.

A pattern (highlighted by the bold) is a group of 3 or more shared amino acids. Colors indicate clusters of related sequences isolated using low pH/DTT and substrate elution protocols. Asterisks indicate the peptides selected for phage ELISA analysis.

Thirty-eight of the 40 plaques isolated from the mixture of the linear libraries had an insert encoding a peptide. Of these, only two encoded 7-mers indicating that the panning conditions favored selection of 12-mers. Further sequence analyses revealed an

enrichment of particular sequences and enabled sorting of the peptides into several groups of identical or related members. Six peptides (NFAAIAPQQFFL, QSTYENPMSPQM, NYSHLRVKLPTP, QMFEGPMSRLHL, DAPMTGRQMSTN and SEPMHRPMSDMQ) were isolated multiple times. The peptide NYSHLRVKLPTP and related peptides were isolated using both substrate and low pH elution protocols. Other clusters contained sequences that shared either four (**Q**IHRT**MT**PF**Q**LI / **Q**PFAL**MT**GN**Q**QS and A**P**M**S**LRH**M**KQ**S**A / G**P**T**S**HLQ**M**YN**S**G) or three amino acids (MMG**P**QPNL**L**RD**Q** / NTW**P**MHRP**L**LN**Q**).

The 40 plaques that were selected from screening the cyclic/constrained peptide library all had inserts. In this case, no single peptide sequence occurred more than once. There was considerable overlap observed in the sequences obtained from the substrate and DTT elution protocols. The longest consensus sequences found were TPLGPL and SXTPVQQ, each present in 2 peptides. However several peptides shared 3 or 4 amino acids (excluding the terminal cysteins) and could be assigned to a particular cluster using a comparison algorithm. In order to compare the 2 panning protocols, we set a cut off at a minimum overlap of 7 amino acids, a minimum match of 3 amino acids and a minimum of 6 sequences sharing that pattern. Interestingly, 13 peptides shared one pair of consensus sequences (PMS, PQQ) and 11 peptides possessed a different pair (APQ, PXPQ).

Eight peptides from either the linear or cyclic phage display libraries, representing each of the major clusters (Table 1 indicated *), were chosen for phage ELISA analysis. The amounts of target (bound either directly to the plate or indirectly via an anti-His-Tag antibody bound to the plate), detection antibody and phage were optimized. We determined that the optimum conditions (data not shown) involved binding Ov-iPGM directly to the plate and using the maximum amount of phage possible, which was dictated by the titers obtained after amplification.

Four linear peptides, one 7-mer (QMTPPMQ) and four 12-mers (QSTYENPMSPQM, QMFEGPMSRLHL, DAPMTGRQMSTN, NFAAIAPQQFFL), demonstrated marginally higher binding to the target compared to the BSA control (Fig 1A). Three of these peptides were obtained from the substrate elution protocol

(DAPMTGRQMSTN, QMTPPMQ, QMFEGPMSRLH). QMFEGPMSRLHL gave the highest values both against the target and BSA, suggesting substantial non-specific binding despite the fact that this peptide was isolated using the substrate elution protocol (figure 1A). Substantially more binding was observed using the peptides isolated from the constrained library (Figure 1B). In fact all the phage tested demonstrated affinity for the target. The binding also appeared more specific since there was higher binding relative to BSA.

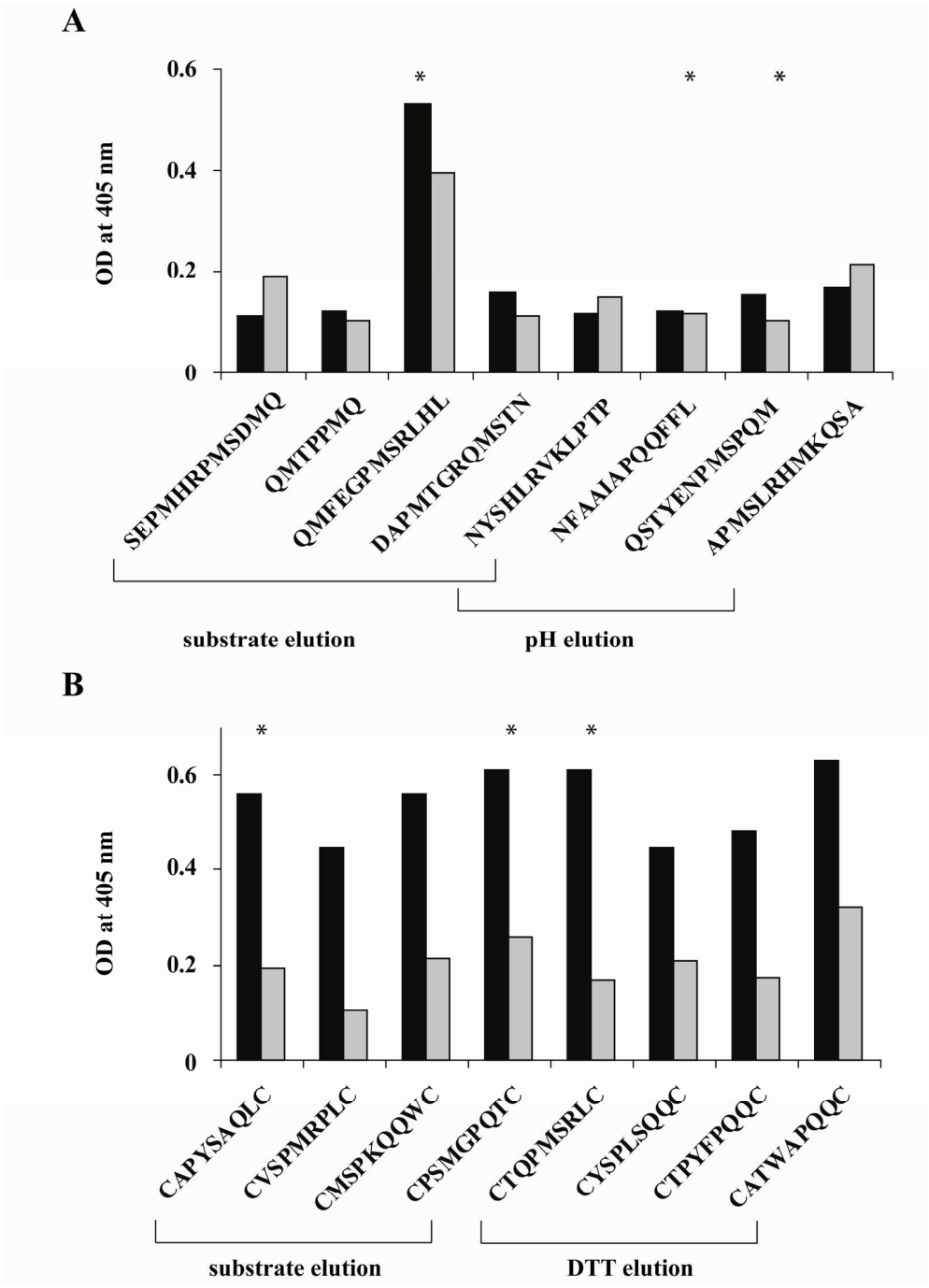

Figure 1. Phage ELISA measurement of binding of selected phage from the linear (A) and cyclic (B) phage display libraries to immobilized Ov-iPGM (black bars). Control plates were coated with bovine serum albumin (grey bars). * indicates the peptides selected for enzyme inhibition studies.

Peptides representing each group (linear: QSTYENPMSPQM, QMFEGPMSRLHL and NFAAIAPQQFFL; and cyclic: CAPYSAQLC, CPSMGPQTC and CTQPMSRLC) were synthesized and analyzed for iPGM inhibitory activity. Each peptide was synthesized with either the amino acids GGGS or GGGC added to the C-terminus of the peptide to mimic the sequence presented at the surface of the phage. The C-terminus of each peptide was amidated, so the global charge of the peptide remained comparable to that found on the phage surface (the carboxylate at the end of the last amino acid would create a negative charge). Two of the peptides (ACAPYSAQLC, NFAAIAPQQFFL) were poorly soluble so they could not be pursued further. The remaining peptides were tested at various concentrations and compared with a control/unrelated peptide (FHENWPS). Unfortunately, even at the highest concentrations tested (100 μM), no inhibitory activity was observed.

In conclusion, three rounds of panning of various phage display libraries led to several consensus peptide sequences. Some peptides were shown to have reasonable affinity for the target in phage ELISA. Several peptides were synthesized and analyzed for enzyme inhibitory activity. Unfortunately none were found to be inhibitors of enzyme function in monovalent form. Future work should include testing cross-linked peptides to enhance avidity and mimic the situation at the surface of the phage, further refinement of the sequences either by further panning or using SPOT synthesis, which enables the production of large arrays of synthetic peptides on planar cellulose support enabling the evaluation of the importance of each amino acid (Reineke *et al.*, 2001; Huang *et al.*, 2003). A sequential elution protocol could be used with an acid elution, allowing elimination of the non-tight binding peptides, followed by substrate elution to release the specific binders.

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Chapter 6: Functional characterization of the unrelated phosphoglycerate mutases of *Escherichia coli*

Chapter 6

Caractérisation fonctionnelle des phosphoglycérates mutases non-apparentées d'*Escherichia coli*

Les phosphoglycérates mutases catalysent l'interconversion du 2- et 3-phosphoglycerate dans les voies glycolytique et néoglucogénique. Bien que ces voies soient hautement conservées parmi différents organismes, deux formes distinctes de PGMs existent, la phosphoglycérate mutase cofacteur-dépendante (PGMd) et l'indépendante (PGMi). Ces enzymes n'ont aucune similarité de séquence amino-acidique et diffèrent dans leur mécanisme de catalyse. Les vertébrés possèdent uniquement la forme PGMd tandis que chez les autres organismes, la distribution des deux formes a toujours été considérée complexe et imprédictible car chaque forme ou les deux peuvent être présente. Dans ce manuscrit nous décrivons le clonage et l'expression de la PGMd et PGMi d'*Escherichia coli* et nous effectuons une analyse du rôle de ces deux enzymes *in vivo*. J'ai surexprimé les deux enzymes chez *E. coli* avec un His-tag en raison du succès obtenu avec les enzymes de nématodes. Dans ce cas, peu d'optimisation fut requis. J'ai ensuite évalué l'activité des enzymes recombinantes et examiné leur dépendance pour le cofacteur 2,3 bisphosphoglycerate et les ions métalliques, ainsi que leur sensibilité au vanadate. Les deux enzymes fonctionnent comme des phosphoglycérates mutases typiques mais l'enzyme cofacteur dépendante (PGMd) a une activité significativement plus haute que la forme cofacteur indépendante (PGMi). Comme il a été reporté que certaines PGMs pouvaient posséder une activité de phosphatase, nous avons teste cette activité mais n'avons pas pu la détecter. Nous avons créé des mutants nuls pour étudier le rôle différentiels de la PGMi et PGMd durant la croissance bactérienne. Un phénotype a été observé pour le mutant PGMd qui poussait lentement en raison d'un retard dans la sortie de phase stationnaire. Surexpression de PGMd ou PGMi dans ce mutant a permis de surmonter le défaut de croissance, fournissant des preuves supplémentaires que les deux enzymes fonctionnent dans le même processus métabolique et sont donc des enzymes analogues. Les souches développées dans cette étude peuvent être utilisées dans un criblage à haut débit pour étudier la spécificité d'inhibiteurs pour l'activité PGM.

Chapter 6

Functional characterization of the unrelated phosphoglycerate mutases of *Escherichia coli*

Phosphoglycerate mutase enzymes (PGM) catalyze the interconversion of 2- and 3- phosphoglycerate in the glycolytic and gluconeogenic pathways. Although these pathways are highly conserved among different organisms, two distinct PGM enzymes are known to exist namely co-factor-dependent (dPGM) and independent (iPGM) phosphoglycerate mutases. The enzymes have no amino acid sequence similarity and differ in their mechanism of catalysis. Vertebrates are known to possess the dPGM form exclusively, whereas in other organisms the distribution of the two forms has been considered complex and unpredictable since either dPGM or iPGM, or both forms may be present. In this paper we describe the cloning and expression of the dPGM and iPGM enzymes from *Escherichia coli*, and perform an analysis of the role of the two enzymes *in vivo*. I over expressed the two enzymes in *E. coli* with a His-tag due to our success with the nematode enzymes. In this case little optimization was required. I then evaluated the activities of the recombinant enzymes and examined their dependence on the cofactor 2,3 bisphosphoglycerate and metal ions, and sensitivity to vanadate. Both enzymes functioned as typical PGMs but the cofactor-dependent enzyme (dPGM) had significantly higher activity than the cofactor-independent form (iPGM). Since it has been reported that certain PGMs may possess phosphatase activity, we also tested but did not detect this activity. We created null mutants to study the differential roles of iPGM and dPGM during bacterial growth. A phenotype was observed for the *dPGM* mutant which grew slowly due to a delay in exiting stationary phase. Overexpression of dPGM or iPGM in this mutant overcame the growth defect, providing further proof that they function in the same metabolic processes and are, therefore, analogous enzymes. The strains developed in this study can be used in high throughput screens for specific inhibitors of iPGM activity.

Chapter 6

Functional characterization of the unrelated phosphoglycerate mutases of *Escherichia coli*

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Résumé

La phosphoglycérate mutase (PGM) inter convertit le 2- et 3-phosphoglycérate durant la glycolyse et la néoglucogenèse. Deux formes de l'enzyme existent mais celles ci n'ont aucune similarité de séquence et ont des structures tridimensionnelles différentes. Une de ces formes appartient à la super famille des phosphatases acides et requiert le cofacteur 2,3-bisphosphoglycerate, tandis que la seconde appartient à la superfamille des phosphatases alcalines et agit indépendamment d'un cofacteur. Bien que la plupart des organismes n'aient qu'une forme de PGM, *Escherichia coli* encode les deux, ce qui soulève des questions quand à leurs rôles respectifs dans cette bactérie. Nous avons produit des enzymes recombinantes afin d'examiner leurs activités en tant que PGM et phosphatase d'ordre générale et nous avons créé des mutants déficients pour étudier leurs rôles différentiels lors de la croissance bactérienne. Les deux enzymes fonctionnent comme des PGMs typiques mais la phosphoglycérate mutase dépendante (PGMd) possède une activité significativement plus haute que la phosphoglycérate mutase indépendante (PGMi). Cette dernière est une métalloenzyme et a montré une activité accrue en présence de manganèse. Il n'y a pas eu de preuve d'activité phosphatase. Un phénotype a été observé pour le mutant *PGMd*, lequel avait une croissance plus lente due à un retard dans la sortie de la phase stationnaire. Surexpression de la PGMd ou PGMi dans ce mutant a permis de surmonter le défaut de croissance, apportant des preuves supplémentaires qu'elles fonctionnent dans le même processus métabolique et sont par conséquences, des enzymes analogues. D'autres génomes entérobacteriens prédisent des orthologues des PGMds et PGMis d'*E. coli*, suggérant que la coexistence peu commune de distinctes phosphoglycérates mutases pourrait être commune dans ce groupe bactérien.

Mots-clés: phosphoglycérate mutase; *Escherichia coli*; glycolyse

Abstract

Phosphoglycerate mutases (PGM) interconvert 2- and 3-phosphoglycerate during glycolysis and gluconeogenesis. Two forms of enzyme exist but show no sequence similarity and have distinct three-dimensional structures. One form belongs to the acid phosphatase superfamily and requires the cofactor, 2,3-bisphosphoglycerate, while the other belongs to the alkaline phosphatase superfamily and acts independently of cofactor. While most organisms have only one form of PGM, *Escherichia coli* contains both, raising questions regarding their relative roles in this bacterium. We produced recombinant enzymes to examine their activities as PGMs and general phosphatases and we created null mutants to study their differential roles during bacterial growth. Both enzymes functioned as typical PGMs but the cofactor-dependent enzyme (dPGM) had significantly higher activity than the cofactor-independent form (iPGM). The latter is a metalloenzyme and showed enhanced activity in the presence of manganese. There was no evidence of phosphatase activity. A phenotype was observed for the *dPGM* mutant which grew slowly due to a delay in exiting stationary phase. Overexpression of dPGM or iPGM in this mutant overcame the growth defect, providing further proof that they function in the same metabolic processes and are, therefore, analogous enzymes. Other Enterobacteriale genomes predict orthologs of *E. coli* dPGM and iPGM, suggesting that the unusual coexistence of distinct phosphoglycerate mutases may be common in this bacterial group.

Keywords: phosphoglycerate mutase; *Escherichia coli*; glycolysis

Introduction

Phosphoglycerate mutase (PGM; E.C. 5.4.2.1.) catalyzes the interconversion of 2- and 3-phosphoglycerate (2-PG and 3-PG) in glycolysis and gluconeogenesis. Despite conservation of these pathways in most organisms, two distinct forms of PGM that have no similarity in primary sequence or three-dimensional structure are known. Cofactor-dependent PGM (dPGM) requires the cofactor, 2,3-bisphosphoglycerate (2,3-BPG), for activity. The dPGM enzymes, having a molecular mass of about 27 kD, are usually active as dimers or tetramers and catalyze the intermolecular transfer of a phosphoryl group between the monophosphoglycerates and cofactor via a phosphohistidine intermediate. Sequence and structural analyses place dPGM in the acid phosphatase superfamily along with enzymes such as fructose-2,6-bisphosphatase and acid phosphatases (Fothergill-Gilmore and Watson, 1989; Jedrzejas, 2000). Cofactor-independent PGM (iPGM) is about 57 kD, active as a monomer, and catalyzes the intramolecular transfer of the phosphoryl group between monophosphoglycerates through a phosphoserine intermediate. The iPGM enzymes belong to the alkaline phosphatase superfamily along with phosphopentomutases and certain sulfatases to name a few (Fothergill-Gilmore and Watson, 1989; Galperin *et al.*, 1998; Jedrzejas, 2000). The two forms of PGM can be distinguished further by the metal ion requirement of iPGM and the sensitivity of dPGM to vanadate (Carreras *et al.*, 1982; Jedrzejas, 2000).

PGM sequences, in particular those of iPGM, are well conserved even across different kingdoms (Jedrzejas, 2000), allowing their identification in genome sequences from diverse organisms. However, since both dPGM and iPGM are members of larger phosphatase superfamilies containing diverse enzymes with related sequences, the identification of PGMs solely by bioinformatic means should be treated with caution. Indeed, a predicted dPGM of *Bacillus* spp. was shown by molecular modeling and enzymatic analyses of recombinant protein to encode a broad specificity phosphatase (Rigden *et al.*, 2001). Small-scale bioinformatic surveys and biochemical studies have revealed that only iPGM is present in plants and nematodes while only dPGM is found in mammals (Carreras *et al.*, 1982; Fraser *et al.*, 1999; Zhang *et al.*, 2004). Within other

phylogenetic groups the distribution of the two PGM forms is complex and has been described as appearing haphazard (Fraser *et al.*, 1999). Most bacteria, protozoa and fungi contain either iPGM or dPGM, although certain bacteria and protozoa contain both forms.

The evolutionary origins of dPGM and iPGM that underlie their unpredictable distribution are not clear (Fothergill-Gilmore and Watson, 1989; Jedrzejas, 2000). Similarly, the respective roles of dPGM and iPGM in organisms that contain both enzymes are uncertain. *Escherichia coli* contains dPGM and iPGM, and in this organism, at least, distinct PGM activities were reported both in crude cell extracts and when expressed in recombinant form (Fraser *et al.*, 1999). Recombinant dPGM (GpmA) showed 10-fold higher catalytic constants than iPGM (GpmM) and the endogenous dPGM activity varied from 5 to 35-fold greater than that of iPGM during the *E. coli* growth cycle even though the molar expression levels of the proteins were broadly similar, raising questions about the role of iPGM in *E. coli*. In this study we generated null mutants of *iPGM* and *dPGM* for phenotypic analysis. We report that loss of dPGM leads to delayed growth both in liquid cultures and on solid medium, apparently due to a delay or defect in exiting stationary phase. We further show that the wild type phenotype can be restored by overexpression of either dPGM or iPGM in *dPGM* mutants. We produced recombinant dPGM and iPGM for detailed biochemical analyses to address the specific PGM and phosphatase activities of each enzyme. Both recombinant proteins exhibited PGM activity and neither appeared to have general phosphatase activity.

Materials and methods

Bacterial strains, media and growth

Recombinant iPGM and dPGM were expressed in *E. coli* T7 Express (New England Biolabs). Deletions of *iPGM* or *dPGM* were made in the *E. coli* K-12 derivative, MG1655 (*E. coli* Genetic Stock Center**).** Bacteria were grown in Luria Bertani (LB) medium and in 3-(N-morpholino) propanesulfonic acid (MOPS) minimal medium ((Neidhardt *et al.*, 1974); TekNova), supplemented with 0.1% glucose. For

production of recombinant proteins or complementation by plasmid constructs, ampicillin (100 μg/ml) was included in the growth medium. Bacterial growth was at 37°C and cultures were shaken at 250 r.p.m.

PGM cloning, expression and enzyme assays.

Full-length *iPGM* and *dPGM* were cloned into the pET-21a vector (Novagen) for expression of recombinant proteins with C-terminal His_6 tags in *E. coli*. For PCR amplification of *iPGM* the following primers were used: 5'-ATAAGTGGATCCATGTTGGTTTCTAAAAAACC-3' and 5'-TAAGTTCTCGAGTTCCACGATGAACAGCG-3', while for *dPGM* the primers were: 5'-ATAAGTGGATCCATGGCTGTAAGCTGGT-3' and 5'-TAAGTTCTCGAGCTTCGCTTTACCCTGGTTTG-3'. BamHI and XhoI restriction sites in each primer pair are underlined. The sequences were amplified from genomic DNA from *E. coli* strain T7 Express using the Expand High Fidelity PCR System (Roche) and the PCR products digested with BamHI and XhoI (New England Biolabs) for cloning into the corresponding sites of pET-21a. Constructs were verified by DNA sequencing before expression of recombinant protein. Optimal expression of both *iPGM* and *dPGM* was achieved following induction with 0.3 mM isopropyl-1-thio-β-D-galactopyranoside (Sigma) for 3 h at 37°C. The His-tagged proteins were extracted and purified on nickel columns (Qiagen) under native conditions according to the manufacturer's instructions.

The purified recombinant proteins were assayed for PGM activity in the glycolytic direction (3-PG to 2-PG) using a standard enzyme-coupled assay as described previously (Raverdy *et al.*, 2007). Briefly, PGM was added to 1 ml assay buffer (30 mM Tris-HCl, pH 7.0, 5 mM $MgSO_4$, 20 mM KCl) supplemented with 0.15 mM NADH, 1 mM ADP, 1.5 mM 3-PG substrate (Sigma P8877) and 2.5 units of each coupling enzyme, namely enolase (Sigma E6126), pyruvate kinase (Sigma P7768) and L-lactic dehydrogenase (Sigma L2518). Reactions were at 30°C for 5 min with data collected every 10 s using a DU 640 spectrophotometer (Beckman). Consumption of NADH at 340 nm provided an indirect measurement of PGM activity since the amount of NADH

converted to NAD corresponds to the amount of reaction product, 2-PG. One unit of PGM activity is defined as the amount of activity necessary to convert 1.0 μmole NADH to NAD per minute under standard assay conditions. The effect of manganese ions was studied by adding manganese chloride to the standard assay buffer to a final concentration of 1 mM. Sensitivity to vanadate was addressed by incubating the recombinant enzymes with different concentrations of sodium metavanadate (Acros) for 15 min prior to the assay. Possible phosphatase activity was assessed in 200 μl reactions using 10 μg enzyme and 50 mM *p*-nitrophenyl phosphate (New England Biolabs) as substrate. Various buffer systems were used: NEBuffer 3, pH 7.9, NEBuffer EcoRI, pH 7.5 (both from New England Biolabs), PGM assay buffer, pH 7.0 (see above), and 1 M diethanolamine, 1 mM $MgCl_2$, pH 9.75. The effect of different metal ions was determined by addition of either $ZnCl_2$ or $CoCl_2$ to these four magnesium-containing buffers. Calf intestinal phosphatase (New England Biolabs) served as an alkaline phosphatase positive control in each buffer. Reactions were incubated at 37^{o}C for 5 min before being stopped by addition of 1 ml 1N NaOH. To monitor possible weak phosphatase activity, some duplicate reactions were incubated for 1 h. The production of *p*-nitrophenylate was determined spectrophotometrically at 405 nm and compared to controls lacking either substrate or enzyme.

Construction and characterization of E. coli PGM mutant strains

Separate strains bearing either a deletion of the entire *iPGM* or *dPGM* open reading frame of *E. coli* MG1655 were prepared by λ Red -mediated recombination (Datsenko and Wanner, 2000). PCR primer pairs were designed such that their 5' ends corresponded to the sequence immediately upstream and downstream of each PGM translational start and stop codon, respectively, while the 3' ends of each primer pair (underlined) corresponded to the P1 and P2 priming sites of the pKD4 plasmid (Datsenko and Wanner, 2000). For targeting *iPGM* the primers used were: 5'TACGCAAATTTTGACTCTTGAGTATGAGGTTGTCCGCAGTGTAGGCTGGAG CTGCTTC-3' and

5’GTCATGGTATTAATCGCCTTTCCCCTCATGGGGAGGGACATATGAATATCCT CCTTAG-3’. For targeting *dPGM* the primers used were: 5’-TCACTTGCAGTCGGCTTTCTCATTTTAAACGAATGACGTGTGTAGGCTGGAGC TGCTTC-3’ and 5’AGAATTATTATCATTAAAAGATGATTTGAGGAGTAAGTATCATATGAATAT CCTCCTTAG-3’. The gene deletions in the resultant strains, MG1655*ΔiPGM::FRT1* and MG1655*ΔdPGM::FRT1*,were confirmed by PCR with diagnostic primers and by DNA sequencing. FRT1 indicates a FLP recombinase recognition site left at each locus after removal of a kanamycin cassette used during strain construction (Datsenko and Wanner, 2000).

The growth of the *ΔdPGM* and *ΔiPGM* strains relative to the MG1655 parental strain was assessed by diluting overnight cultures grown in MOPS minimal medium supplemented with 0.1% glucose into 10 ml fresh LB medium in Nephelo sidearm flasks (Bellco Biotechnology) to give initial OD_{600} values of 0.03. Each strain was grown in triplicate and turbidity monitored using a photoelectric colorimeter (Klett Summerson**).** For evaluating growth on solid media, overnight cultures grown in MOPS containing 0.1% glucose were standardized to similar optical density when necessary then serially diluted and 100 µl of each dilution plated in quadruplicate to LB agar. The number of colonies on each plate was recorded after overnight growth.

Complementation of ΔdPGM

To examine whether *E. coli iPGM* or *dPGM* could complement the *ΔdPGM* growth phenotype, these genes were cloned into the pKK223-3 expression vector (Amersham Pharmacia Biotech) and transformed into the *ΔdPGM* mutant strain. The following primers were used for amplification of *dPGM*: 5’-GAACTCGAATTCCATGGCTGTAACTAAGCTGGT-3’ and 5’-TAAGTTGAATTCTTACTTCGCTTTACCCTGGTTTG-3’. For amplification of *iPGM* the primers were: 5’-ATAAGTGAATTCATGTTGGTTTCTAAAAAACC-3’ and 5’-TAAGTTGAATTCTTATTCCACGATGAACAGCG-3’. The position of an EcoRI site in each primer is underlined. The sequences were amplified from the pET-21a

constructs described above using Phusion High Fidelity DNA polymerase (New England Biolabs). The PCR products were digested with EcoRI, then cloned into the corresponding site of pKK223-3 and verified by DNA sequencing. The constructs were designated pKK*iPGM* and pKK*dPGM*. For complementation assays, strains MG1655, *ΔdPGM*, and *ΔdPGM* harboring, separately, pKK*iPGM* and pKK*dPGM* were initially grown overnight in MOPS containing 0.1% glucose. These cultures were then serially diluted and plated in triplicate to LB agar as described above. Strain *ΔdPGM* harboring empty plasmid, designated pKK, served as a control.

Results

Expression and activity of E. coli dPGM and iPGM

Recombinant dPGM and iPGM were produced in *E. coli* to permit enzymatic analysis. Both proteins were abundantly overexpressed and subsequently purified by nickel-nitrilotriacetic acid chromatography. The yield of each protein was in excess of 300 mg per liter. Imidazole (100 mM for iPGM; 200 mM for dPGM) in the elution buffer resulted in release of the proteins from the nickel resin with a high degree of purity (Figure 1). The sizes of dPGM and iPGM bearing vector-encoded N-terminal T7 and C-terminal His_6 tags are consistent with their calculated molecular masses of 31 kD and 58.6 kD, respectively.

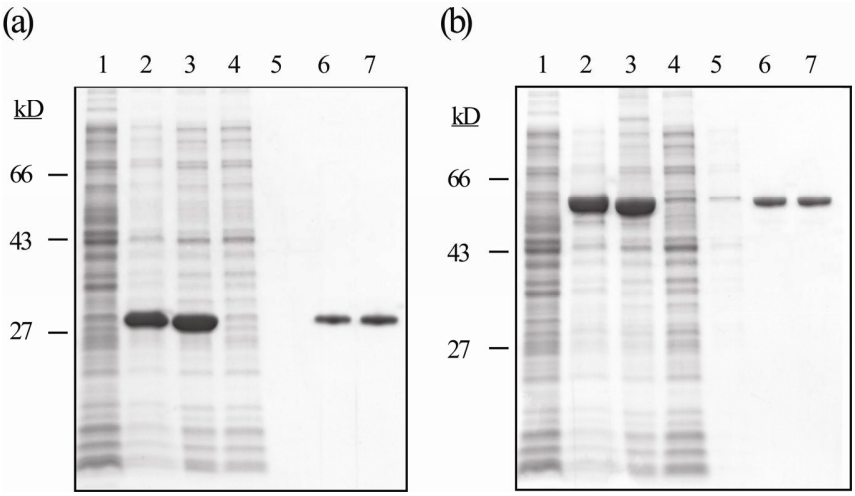

Figure 1: Overexpression and purification of recombinant dPGM (a) and iPGM (b).

Lanes: 1, *E. coli* total protein without induction with IPTG; 2, *E.coli* total protein following induction with IPTG; 3, soluble *E. coli* proteins after cell disruption; 4, flow-through from the nickel column; 5, Wash of nickel column prior to elution; 6 and 7, elution fractions from nickel column using imidazole (200 mM for dPGM, 100 mM for iPGM).

Both *E. coli* enzymes exhibited PGM activity as evidenced by the consumption of NADH by the coupling enzymes used in the assay (Figure 2). The slope of the curves in Figure 2 was used to calculate PGM specific activities of ~ 1.8 units/mg and 229 units/mg for iPGM and dPGM, respectively. Addition of 1 mM manganese to the assay buffer resulted in more than a 4-fold increase in iPGM activity (Figure 2) yielding a specific activity of ~8 units/mg. The activity of dPGM was unaffected by the addition of manganese as expected (data not shown) since dPGM enzymes are not metalloenzymes (Jedrzejas, 2000), however it was sensitive to vanadate, a known inhibitor of dPGM (Carreras *et al.*, 1980), with an IC50 of 0.65 mM (data not shown). The activity of dPGM was determined in the absence of 2,3-BPG, since inclusion of this cofactor did not enhance activity (data not shown). Commercially available 3-PG, the substrate for PGM assays in the glycolytic direction, contains 2,3-BPG as a contaminant in sufficient amounts to stimulate dPGM activity causing an apparent lack of dependency on cofactor (Johnsen and Schonheit, 2007).

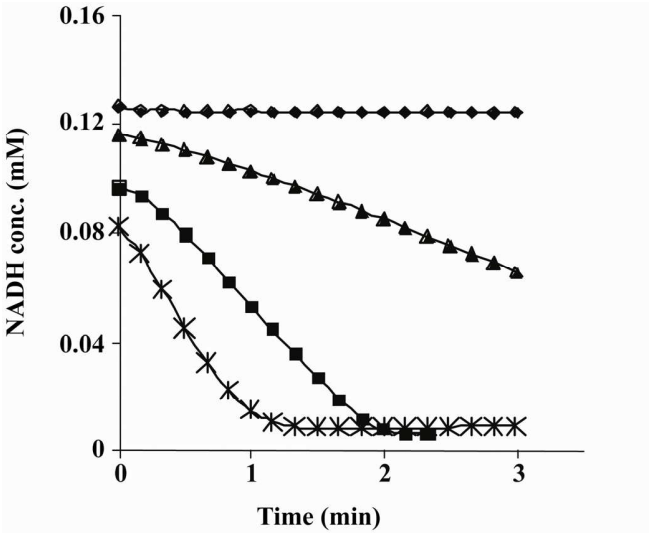

Figure 2: PGM activity of recombinant dPGM and iPGM.

Conversion of 3-PG to 2-PG by 0.25 µg dPGM (■) and 10 µg iPGM (σ) assayed in standard, magnesium-containing buffer. Conversion of 3-PG to 2-PG by iPGM in buffer supplemented with 1 mM manganese chloride is shown for comparison (✱). A control lacking any recombinant protein is also shown (◆). Conversion of 3-PG to 2-PG is determined indirectly by a decrease in NADH concentration as measured by its absorbance at 340 nm. Consumption of NADH is directly proportional to PGM activity.

Recombinant iPGM and dPGM were assayed for phosphatase activity against the phosphatase substrate, *p*-nitrophenyl phosphate in buffers of different pH values and in the presence of different metal ions. No phosphatase activity was detected for either enzyme whereas calf intestinal phosphatase was active under all conditions tested (data not shown).

Characterization of ΔiPGM and ΔdPGM mutant strains

Strains deleted for each of the predicted PGM genes were made in the wild-type *E. coli* K-12 strain, MG1655, using established methodology (Datsenko and Wanner, 2000). Repeated attempts to create a *ΔiPGM*, *ΔdPGM* double deletion by targeting the remaining locus in each of the mutant strains were unsuccessful. Neither mutant strain exhibited an obvious phenotype when LB medium was inoculated from overnight cultures in LB (data not shown). However, when MOPS minimal medium supplemented

with glucose was used, either for initial overnight culture or for subsequent growth analysis or for both, a phenotype was reproducibly observed for the *ΔdPGM* strain (Figure 3a). This strain showed a delay in exiting stationary phase relative to *ΔiPGM* and MG1655. However, the calculated doubling times for both mutants and the MG1655 parent were similar during logarithmic growth. Similar results were obtained using *iPGM* and *dPGM* transposon insertion mutants (data not shown) supplied by Dr F. Blattner, University of Wisconsin. The phenotype observed in liquid cultures prompted investigation of the colony forming ability of the three strains on solid media. When overnight cultures in minimal medium were serially diluted then plated to LB agar a more striking phenotype was observed (Figure 3b and c) with *ΔdPGM* failing to form colonies after 24 h growth. After an additional 24 to 48 h growth, colonies comparable in number to those observed for *ΔiPGM* and MG1655 were evident on the plates containing the *ΔdPGM* mutant but they were very small and grew slowly (data not shown). This phenotype of *ΔdPGM* on solid medium is in agreement with that observed in liquid culture and is consistent with a delay in exiting stationary phase. This conclusion is supported by the finding that *ΔdPGM* grew comparably to the MG1655 parent when logarithmic phase cultures were diluted and plated on solid medium (data not shown). No phenotype was observed for the *ΔiPGM* mutant strain in these studies.

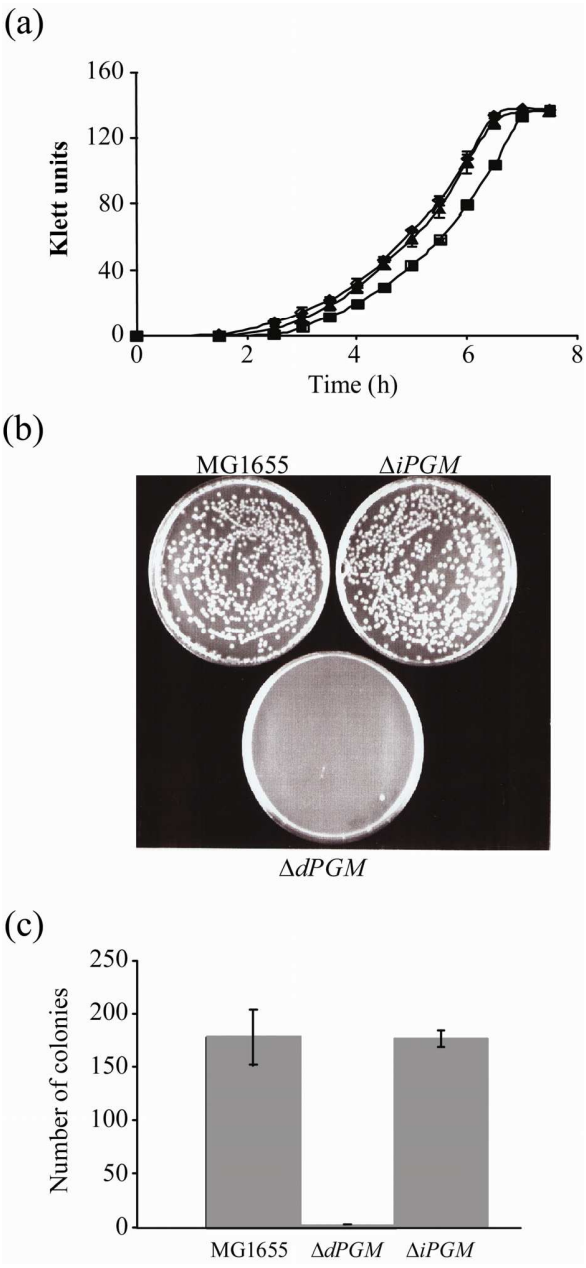

Figure 3: Phenotypes of *ΔdPGM* and *ΔiPGM* mutant strains.

Panel a: Parental wild-type MG1655 *E. coli* (◆) and *ΔdPGM* (■) and *ΔiPGM* (σ) mutant strains grown in minimal medium overnight were inoculated into 10 ml fresh minimal medium to give initial OD_{600} values of 0.03. Growth was monitored by determining turbidity (Klett units) during incubation at 37°C. Each data point represents the mean Klett value of triplicate cultures (± S.D.). **Panels b and c:** Overnight MOPS minimal medium cultures of parental wild-type MG1655 *E. coli* and the *ΔdPGM* and *ΔiPGM* mutant strains were serially diluted in minimal medium and 100 µl of each dilution plated to LB agar. Cells were grown at 37°C and the number of colonies counted. Each dilution of each strain was plated in quadruplicate. Representative plates at 1 x 10^{-5} dilution are shown (b) and the mean numbers of colonies (± S.D.) per plate at 1 x 10^{-6} dilution are plotted (c).

Complementation of ΔdPGM by dPGM and iPGM

The observed phenotype of Δ*dPGM* grown on agar provided a system for complementation experiments using expression constructs transformed into this mutant strain. Plasmids pKK*iPGM* and pKK*dPGM* were introduced into the *ΔdPGM* strain and plated on LB agar after overnight growth in MOPS minimal medium. Strains MG1655, *ΔdPGM* and *ΔdPGM* harboring empty plasmid (pKK) were grown in parallel. Both expression constructs, pKK*iPGM* and pKK*dPGM*, complemented the *ΔdPGM* mutation such that the number of colonies formed after overnight growth was comparable to the parental MG1655 (Figure 4). No colonies were evident when *ΔdPGM* was transformed with the empty vector, pKK (data not shown).

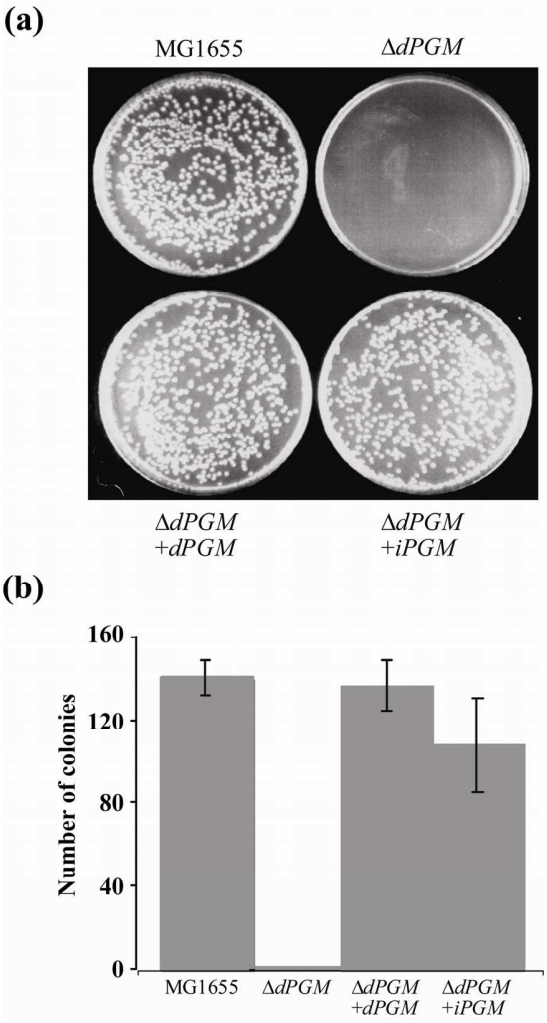

Figure 4: Complementation of the *ΔdPGM* phenotype by *dPGM* and *iPGM*.
Overnight minimal medium cultures of parental wild-type MG1655 *E. coli*, the *ΔdPGM* mutant, and the *ΔdPGM* mutant carrying either the plasmid pKK*iPGM* or pKK*dPGM* were serially diluted in MOPS minimal medium. Triplicate aliquots of 100 µl of each dilution were plated to LB agar and the number of colonies counted after incubation at 37^{o}C. Strains harboring plasmid constructs were grown in the presence of 100 µg/ ml ampicillin. Representative plates at 1 x 10^{-5} dilution are shown (a) and the mean number of colonies (± S.D.) per plate at 1 x 10^{-6} dilution are plotted (b).

Discussion

Although dPGM and iPGM catalyze the same metabolic reactions, the two proteins have no sequence or structural similarities and they use dissimilar catalytic mechanisms. While the majority of organisms possess only iPGM or dPGM, some contain both forms, raising questions concerning their respective functions in the cell. To address these issues we exploited the genetic tractability of *E. coli* to perform a detailed study of the roles of dPGM and iPGM in this organism that contains both forms of PGM.

Initially we overexpressed the predicted dPGM and iPGM enzymes in recombinant form. Both proteins were abundantly expressed and subsequently purified in good yield with a high degree of purity. Both showed typical PGM activities in standard enzyme assays with dPGM exhibiting a specific activity at least an order of magnitude greater than that of iPGM. This result is in agreement with a report of the significantly higher activity of *E. coli* dPGM compared to iPGM (Fraser *et al.*, 1999). However, in both studies, iPGM activity was determined in buffer containing magnesium yet manganese appears to be the preferred ion for bacterial iPGM enzymes that have been characterized (see (Jedrzejas and Setlow, 2001) for review). Indeed, we demonstrated that *E. coli* iPGM has greater activity in buffer supplemented with manganese than in the standard buffer that contains only magnesium. Somewhat surprisingly, the activity was also enhanced when assayed in the presence of cobalt (data not shown). *Clostridium perfringens* iPGM has higher activity with cobalt than with manganese although biochemical evidence suggests that the latter ion is used *in vivo* (Chander *et al.*, 1998). Probably manganese, rather than cobalt, is the physiologically relevant ion for *E. coli* iPGM also since it has been found integrally bound in this enzyme (Fraser *et al.*, 1999) and is the more abundant ion in the cell (Finney and O'Halloran, 2003). Although we demonstrated that certain ions enhanced iPGM activity, the level of activity was still significantly lower than that of dPGM. This relatively low specific activity of *E. coli* iPGM may not result directly from the coexistence of dPGM since bacterial iPGM enzymes can be of low activity (~ 1 unit/mg or less) even in species that lack dPGM (Kuhn *et al.*, 1993; Leyva-Vazquez and Setlow, 1994; Chander *et al.*, 1999). This is in

contrast to eukaryotic iPGMs where specific activities are typically in the range of 50 to 400 units/mg (Chevalier *et al.*, 2000; Guerra *et al.*, 2004; Zhang *et al.*, 2004). However, since most bacterial iPGMs have been assayed from partially purified proteins, the reported low specific activities may not correlate well with those of pure enzyme.

Bioinformatic analyses have suggested the presence of both dPGM and iPGM in *Bacillus subtilis* (Kunst *et al.*, 1997; Galperin *et al.*, 1998; Fraser *et al.*, 1999). However, unlike the situation in *E. coli*, it appeared that iPGM accounted for the major PGM activity while the predicted dPGM had little or no activity (Chander *et al.*, 1999; Pearson *et al.*, 2000). Further studies determined that the predicted dPGM was a broad specificity phosphatase (Rigden *et al.*, 2001), a member of the acid phosphatase superfamily to which dPGM belongs. Deletion of *B. subtilis* iPGM resulted in a severe growth phenotype and asporulation (Leyva-Vazquez and Setlow, 1994) while deletion of the phosphatase had no effect (Pearson *et al.*, 2000). The possibility that *E. coli* iPGM might also function as a phosphatase has been suggested (Fraser *et al.*, 1999) but we were unable to detect any such activity despite using buffers frequently used for determination of alkaline phosphatase activity and testing the various preferred metal ions (Mg^{2+}, Co^{2+}, Zn^{2+}) of bacterial alkaline phosphatases (Wojciechowski and Kantrowitz, 2002). The finding of manganese bound to *E. coli* iPGM (Fraser *et al.*, 1999) is further support for its function as a PGM (Jedrzejas and Setlow, 2001) rather than an alkaline phosphatase. Thus, the confirmed PGM activity of *E. coli* iPGM and the apparent lack of phosphatase activity contrast the observations reported for the two predicted PGMs of *Bacillus spp*. and indicate that both *E. coli* iPGM and dPGM function as PGMs although additional cellular functions can not be ruled out.

To further characterize the roles of dPGM and iPGM in *E. coli* we constructed null mutants of each. Analysis of the *ΔdPGM* and *ΔiPGM* strains revealed a growth phenotype for the former but no phenotype for the latter consistent with the observed higher PGM activity of dPGM. The iPGM activity is apparently sufficient to support growth of *ΔdPGM* cells, although this mutant showed less robust growth than that of wild type *E. coli*. The phenotype appeared to be related to stationary phase since *ΔdPGM* cells grown in liquid media showed a longer lag phase but had doubling times similar to

ΔiPGM and parental wild type *E. coli*. A similar delayed growth phenotype was observed when overnight cultures were diluted and plated on LB agar. No *ΔdPGM* colonies formed after overnight incubation whereas *ΔiPGM* and wild type cells grew normally. Tiny *ΔdPGM* colonies only became apparent after an additional 24 to 48 hours of incubation. During stationary phase, energy metabolism is limited and primarily consists of pathways that scavenge potential nutrients from the medium and from within the cell (Huisman *et al.*, 1996). However, upon a return to glucose-containing medium the pathways of central metabolism need to be upregulated to permit rapid growth. This lag phase during which the cell adjusts to the new conditions is extended in *ΔdPGM* cells, presumably because they also have to compensate for the absence of the major PGM activity in their glycolytic pathway. Our observation of a growth phenotype in *E. coli* lacking dPGM is supported by reports from other bacteria such as *Pseudomonas syringae*, a tomato pathogen, where mutation of iPGM, the only PGM form present, resulted in failure to grow and to elicit disease symptoms on the host plant (Morris *et al.*, 1995). Similarly, *B. subtilis* grew extremely slowly and failed to sporulate following iPGM deletion (Leyva-Vazquez and Setlow, 1994). Studies of PGM null mutants or gene transcript reduction by RNAi in eukaryotes such as yeast, protozoa and nematodes lend further support to the essentiality of PGM in these organisms (Rodicio and Heinisch, 1987; Zhang *et al.*, 2004; Djikeng *et al.*, 2007).

The observed *ΔdPGM* growth phenotype could be restored to wild type by dPGM expressed from the plasmid pKK*dPGM* as expected. Interestingly, plasmid pKK*iPGM* also complemented the *ΔdPGM* deletion. This result indicates that while expression of the chromosomal copy of *iPGM* alone is not sufficient to fully compensate for the lack of dPGM activity, the expression of additional iPGM from a medium copy plasmid can restore the mutant cells to normal growth characteristics. It further confirms that iPGM and dPGM can function in the same metabolic pathways.

Thus it appears that in *E. coli*, the *dPGM* and *iPGM* genes both encode active PGMs with dPGM having the major activity. Accordingly, only deletion of *dPGM* results in a detectable phenotype with *ΔdPGM* cells growing slower than *ΔiPGM* and wild type cells due to a delay in exiting stationary phase. This growth phenotype can be

complemented by overexpression of either dPGM or iPGM, confirming shared roles for the two proteins in *E. coli* and establishing them as analogous enzymes. Bacteria with both a predicted dPGM and iPGM are most abundant in the γ-proteobacteria, particularly in the Order Enterobacteriales, where the various *E. coli*, *Salmonella spp*., *Shigella spp*. and *Yersinia spp*. genomes predict the presence of both forms. Therefore, the conclusions drawn from the present study in *E. coli* may have wider implication.

Acknowledgements

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General Discussion and Conclusions

Parasitic nematode diseases are a huge burden for developing countries. Lymphatic filariasis (elephantiasis) and onchocerciasis (river blindness) are two of the most disfiguring and debilitating amongst them. Control of these diseases relies heavily on the use of drugs, often through mass drug administration (MDA) (Woods *et al.*, 2007), and in some areas there are also efforts to interrupt transmission by attempting to control the insect vector population. Three drugs are currently used to treat/control filarial infection namely ivermectin, albendazole, and diethylcarbamazine. Unfortunately, these drugs are inadequate as they are most effective against the larval stages of the parasite and not the adults. No vaccines are available and drug resistance is on the rise (Waghorn *et al.*, 2006; Prichard, 2007). There is therefore an urgent need for better and new drugs in order to eliminate these diseases. Our team has developed a genomic filtering approach to look for new drug targets in nematodes (Kumar *et al.*, 2007). Using this method, independent phosphoglycerate mutase was identified as a top ranking, potential drug target. Phosphoglycerate mutases catalyze the interconversion of 2 and 3-phosphoglycerate in the glycolysis and gluconeogenesis. The same reaction can be catalyzed by two unrelated enzymes, dependent phosphoglycerate mutase (dPGM) and independent phosphoglycerate mutase (iPGM). dPGM is found in mammals but not in nematodes whereas iPGM is found in nematodes but not in mammals. Previous work in our lab has shown that iPGM is required for normal development of the free-living, model nematode *C. elegans* (Zhang *et al.*, 2004).

The first study presented here describes the cloning and expression of *O. volvulus* iPGM, as well as the detailed biochemical characterization of three nematode iPGMs from *O. volvulus*, *B. malayi* and *C. elegans*. We showed that the three iPGMs possess a high level of sequence similarity to each other (higher than 70%) and with the previously described iPGMs from other organisms. Like other iPGMs, the nematode enzymes were also found to have a dependence on metal ions. We determined that magnesium and manganese ions were the preferred ions, in contrast to the case for the well characterized enzyme from the protozoan parasite *T. brucei* iPGM which prefers cobalt ions (Collet *et al.*, 2001). Further characterization revealed that the three nematode enzymes possess the classical features of iPGM enzymes in that they are insensitive to vanadate and are not

activated by the cofactor 2,3 bisphosphoglycerate, unlike dPGMs. The results of this study indicate that the nematode iPGMs share similar enzymatic characteristics and indicate that a single inhibitor would be likely be effective against all these enzymes. The close similarity of the filarial parasite enzymes with the iPGM from *C. elegans* supports the use of *C. elegans* in whole worm screens to evaluate the efficacy of specific inhibitors.

A number of filarial nematodes, including those responsible for lymphatic filariasis and onchocerciasis are infected with a bacterial endosymbiont, *Wolbachia*. The bacteria is a mutualist endosymbiont and is considered a drug target (Taylor *et al.*, 2005; Hoerauf and Pfarr, 2007) since disruption of *Wolbachia* by antibiotics (mainly the tetracycline family) results in death of the host parasite (Hoerauf *et al.*, 2001; Hoerauf *et al.*, 2003a; Hoerauf *et al.*, 2003b). The genome sequence of *Wolbachia* from *B. malayi* has recently become available (Foster *et al.*, 2005). Genomic analysis of that organism led us to discover the presence of a putative iPGM. The second study presented here, describes the cloning and expression of this putative iPGM. Recombinant *w*Bm-iPGM was difficult to produce because of its low levels of expression and solubility, and lack of activity. Several expression systems were explored for bacterial expression (*E. coli*), as well as various *E. coli* strains, without success. Expression issues were resolved by using a *K. lactis* expression system that allowed us to produce and evaluate the activity of the recombinant enzyme. Our results demonstrate that the *Wolbachia* endosymbiont also possesses an active iPGM which warrants further investigation as a drug target.

Our studies on nematode and *Wolbachia* iPGM enzymes resulted in a collaboration with a group working on the procyclic stage of the protozoan parasite *Trypanosoma brucei*. *T. brucei*, is the parasite responsible for human African trypanosomiasis or sleeping sickness, and was previously reported to have iPGM (Chevalier *et al.*, 2000). The third study presented describes the results of an investigation of the role of iPGM during the growth of procyclic *T. brucei in vitro*. The genome sequence predicted an iPGM encoding a protein with a C-terminus that was different from a *T. brucei* iPGM sequence published earlier (Chevalier *et al.*, 2000). I cloned and expressed protein corresponding to the new sequence and demonstrated that it

encoded an active iPGM. We then established parasite cell lines with inducible expression of double stranded RNA corresponding to iPGM to knock down the gene activity by RNAi. I measured levels of PGM activity in extracts from cell lines, and we showed that reduction in enzyme activity correlated with a reduction in the steady state levels of iPGM mRNA levels. We also showed that cell growth was dependent on a normal iPGM expression thus proving that the iPGM is required for growth and thus worthy of further study as a drug target in the protozoan *T. brucei*. These results are in agreement with those obtained by another team working on the blood stream form of the parasite (Albert *et al.*, 2005).

After discovering the presence of an active iPGM in *Wolbachia* we performed further genomic analysis on the glycolysis/gluconeogenesis pathways in this organism. We were particularly interested in identifying other enzymes that are not found in mammals. The conversion of phosphoenolpyruvate to pyruvate can in nature be catalyzed by two enzymes namely pyruvate kinase (PK), which is the enzyme found in the majority of organisms, and pyruvate phosphate dikinase (PPDK) which is absent from mammals. Our analysis revealed that *Wolbachia* lacked PK but possessed a putative PPDK. The fourth study presented describes the cloning, expression and activity of the putative pyruvate phosphate dikinase from *Wolbachia*. The expression and purification of the enzyme was challenging due to its poor stability, however after optimization of the expression and purification protocols, we managed to obtain highly purified active protein. This enzyme has been reported as a potential drug target in a small number of parasitic organisms namely *Entamoeba histolytica* and *Giardia lamblia*, as well as in *T. brucei*. Interestingly, these parasites possess both PK and PPDK, and PPDK appears to be localized in the glycosome compartment, where it would have an essential role in pyruvate metabolism (Acosta *et al.*, 2004).

Our studies have indicated that iPGM and PPDK are potential drug targets in filarial parasites and/or their *Wolbachia* endosymbiont. Our next goal was to identify inhibitors of these enzymes. We chose to explore phage display technology for this purpose since it is readily available at New England Biolabs. Phage display is a powerful method for identifying peptides that specifically bind to a protein through analysis of a

large number of random peptides expressed at the surface of a phage (Smith and Petrenko, 1997). Selection of peptides is performed against a target and the eluted phage can then be sequenced to link the nucleotide sequence to the peptide displayed (Barbas *et al.*, 2001). During my studies I used three libraries displaying random 7-mers (linear or cyclic) or 12-mers to identify peptides which bind to *O. volvulus* iPGM. Several binding peptides were found with consensus sequences, however unfortunately no inhibitory activity could be detected under the conditions used.

Our studies on parasite/*Wolbachia* PGM enzymes led us to a more general interest in the distribution of iPGM and dPGM enzymes in nature, and in pathogens in particular. Some organisms such as yeast and mammals possess the dependent form (Carreras *et al.*, 1982; Fothergill-Gilmore and Watson, 1990), while others like nematodes possess the independent form. Some bacteria such as E. *coli* encode both forms of the enzyme (Fraser *et al.*, 1999). We became interested in the respective roles of dPGM and iPGM in *E. coli*. In the last study, we describe the cloning and expression of both *E. coli* proteins, and evaluated their relative PGM activities, as well as possible phosphatase activities since this had previously been proposed (Rigden *et al.*, 2001). Both enzymes expressed well and showed PGM activity in our assay. The dependent form was shown to be inhibited by vanadate. Neither enzyme displayed any phosphatase activity under the conditions tested. We then created null mutants for each enzyme and observed a growth defect in the mutant lacking dPGM, due to a delay in exiting the stationary phase. That defect could be corrected by over expression of dPGM and iPGM. The two null strains produced could be useful in high throughput screening for inhibitors, and evaluating their specificities.

The mining of genomic information to aid in the discovery of new drug targets in filarial parasites is in its infancy. The work described in this thesis demonstrates that a genomic filtering method can be used to identify 'pre-validated' drug targets. In the process of developing iPGM as a new drug target in filarial parasites, I produced a panel of recombinant, active iPGM (*E. coli*, *T. brucei*, *C. elegans*, *B. malayi*, *O. volvulus* and *Wolbachia*) and dPGM (human and *E. coli*) enzymes from a number of species. I also produced active PPDK from *Wolbachia* and *T. brucei*. This panel of enzymes will aid in

the search for enzyme inhibitors as well as evaluation of their specific activities. Figure 21 illustrates a possible pathway for screening for inhibitors of iPGM using these recombinant enzymes. The method can be adapted to any target. The phage display method that was used to identify peptides which bind *O. volvulus* iPGM illustrates one approach to identifying enzyme inhibitors.

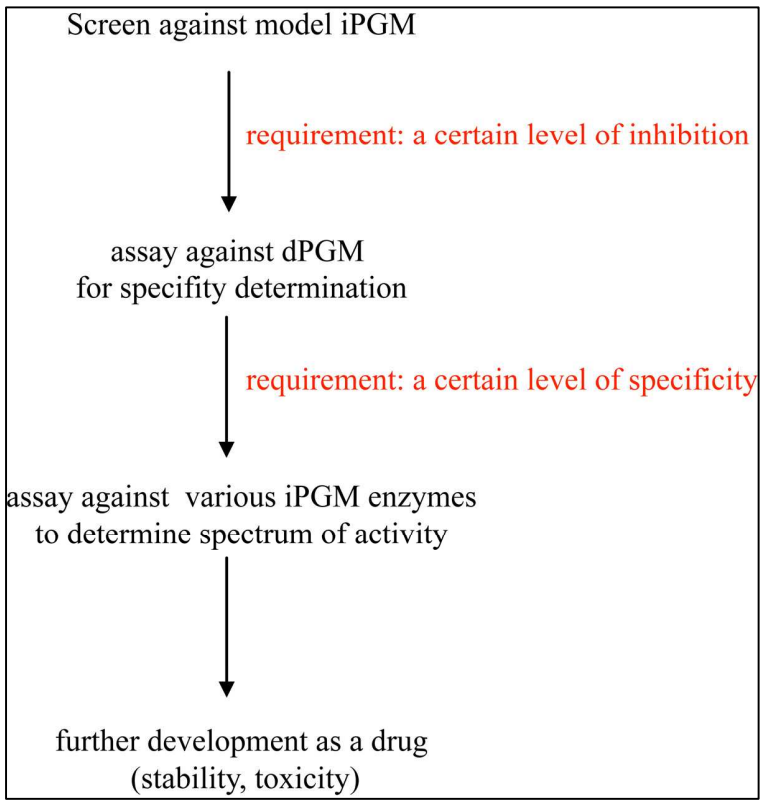

Figure 21: A screening pathway for the discovery of specific inhibitors of iPGM.

Our work justifies targeting glycolysis in filarial drug discovery. Interestingly, one of the drugs currently in use namely albendazole indirectly targets carbohydrate metabolism and glycolysis. Albendazole inhibits glucose uptake through interruption of vesicular transport via the microtubules (Martin *et al.*, 1997), forcing the parasite to use its glycogen reserves and ultimately leading to death. Furthermore, recent analysis by our group (personal communication) of the Database of Essential Genes (DEG) containing data from the screening of 10 bacterial genomes for essential genes (Zhang

and Zhang, 2007), has revealed that glycolytic enzymes are also essential in a number of bacteria. This lends support to exploring energy metabolism in *Wolbachia* as a target for drug intervention.

During my Ph.D studies, I developed methods to produce active, recombinant enzymes from the filarial nematodes and filarial *Wolbachia*. This involved a substantial amount of optimization with the more challenging proteins. Various expression systems were explored, as well as the use of different expression conditions and strains. Purification protocols also had to be optimized. Information in the literature on expression of these enzymes from other organisms was also taken into consideration and specialists in the area of protein expression were consulted. The knowledge gained will be useful in the production of other potential drug targets. The detailed characterization performed on the various enzymes will allow the design of assays for high throughput screens. The optimization of conditions for enzyme activity will facilitate identification and characterization of inhibitors. Taken together, these studies further our understanding of glycolysis/gluconeogenesis in a diverse group of organisms.

Materials and Methods

Standard molecular biology techniques

Purification of plasmid DNA

Plasmids were amplified overnight at 37°C after transformation in *E. coli*. The culture was pelleted and the pellet was processed using the Qiagen miniprep kit in order to obtain high quality DNA.

Cloning

- Digestion

DNA (plasmid and insert) was digested by restriction enzymes from New England Biolabs, following the manufacturer's instructions specific to each enzyme.

- Purification of fragments

The DNA fragments (vector and insert) were purified with the Qiaquick purification kit from Qiagen following the manufacturer's instructions.

- Ligation

Ligations were performed using the Quick ligation kit from NEB following the manufacturer's instructions. Vector and insert were mixed in the quick ligation buffer 1X with a ratio of 1:3. 1µl of QuickT4 DNA ligase was added and the mix was incubated at room temperature for 15 min before transformation.

- Transformation

Competent cells were thawed on ice, 1 µl of ligation or 1µl of plasmid was incubated with the cells for 5 min on ice. The cells were heat shocked at 37°C for 2 min, then incubated on ice for 10 min. Recovery was performed by adding 1 ml of LB medium to the cells. The solution was incubated at 37°C for 1H and 100 µl of cells was plated on the appropriate solid medium. The plates were incubated overnight at 37°C.

Culture Media

Luria Bertani Broth

This medium is classified as a complex medium for bacterial expression of recombinant proteins. 1 l of LB contains 10 g of tryptone, 5 g of yeast extract and 10 g of NaCl at pH 7 (Sambrook *et al.*, 1989). According to the vector systems and the bacterial strains used, different antibiotics were added following standard concentrations (100 μg/ml ampicillin, 35 μg/ml chloramphenicol, 50 μg/ml tetracycline).

YP-glu/gal

This medium is classified as a complex medium for yeast growth. 1 l of YP-Glu medium contains 10 g yeast extract, 20 g bacto-peptone in 950 ml deionized water. 50 ml of a 40% solution of glucose or galactose were added in sterile conditions.

Yeast Carbon Base* (YCB) *agar medium with acetamidase

This medium is classified as synthetic and is used for the selection of yeast transformants. The composition for 1 l is the following: 30 mM Tris-HCl Buffer pH7, 11.7 g YCB medium powder (Difco #239110) 20 g Bacto agar (Becton Dickinson #214050) in 990 ml deionized water. After autoclaving, 5 mM of acetamide were added. YCB agar medium contains glucose and most nutrients (except a nitrogen source) needed to sustain growth of *Kluyveromyces lactis* GG799 Competent Cells. Acetamide is added as a source of nitrogen and is converted to ammonia by acetamidase (the product of the amdS gene present in pKLAC1).

Protein expression

Expression vectors

- pET21a (Novagen)

This plasmid was used for the expression of heterologous protein in *E. coli* under the control of the T7 polymerase promoter; the expressed protein contains a His_6-Tag at the C-terminus. Expression was induced by IPTG.

- pET28a (Novagen)

This plasmid was used for the expression of heterologous protein in *E. coli* under the control of the T7 polymerase promoter, the expressed protein contains a His_6-Tag at the N-terminus. Expression was induced by IPTG.

- Impact vector (New England Biolabs)

The pTWIN1 vector was used for the expression of heterologous protein in *E. coli* under the control of the T7 polymerase promoter. The expressed protein is fused at the N-terminus to a mini intein from the *Mycobacterium xenopi gyrA* gene. Expression was induced by IPTG.

- pKLMF-EK/FX (New England Biolabs)

The pKLAC2 vector was modified to allow intra-cytoplasmic expression of heterologous protein in *K. lactis*. The α-mating factor secretion leader sequence was partially removed by digestion with HindIII and XhoI and replaced with maltose binding protein (MBP). Following cloning in *E. coli*, the plasmid was linearized and inserted by homologous recombination in the *K. lactis* genome. Protein expression is under control of the LAC4 promotor and thus, yeast were grown in YP-gal to produce N-terminal MBP tagged proteins. An Enterokinase (EK) or Factor Xa (FX) sequence cleavage site was inserted after the MBP.

Strains

Bacterial Strains

The prototrophic *E. coli* strain ER2566 (fhuA2 lacZ::T7 gene1 [lon] ompT gal sulA11 R(mcr-73::miniTn10--TetS)2 [dcm] R(zgb-210::Tn10--TetS) endA1 D(mcrC-mrr)114::IS10) is a protein expression strain that allows expression of protein under the control of the T7 promotor, due to the presence of T7 polymerase integrated into the genome.

The prototrophic *E. coli* strain ER2575 is the strain ER2566 carrying the pLysS plasmid. pLysS carries the T7 lysozyme gene, which inhibits the activity of T7 RNA polymerase. pLysS is used to reduce the basal level of gene expression in pET or other T7 vectors.

The prototrophic *E. coli* strain RIL is the strain ER2566 containing the RIL plasmid. The RIL plasmid is a derivative of the p15A replicon that carries the *E. coli argU*, *ileY*, and *leuW* genes, which encode the cognate tRNAs for AGG/AGA, AUA, and CUA codons, respectively. These codons are rarely used in *E. coli*, but occur frequently in ORFs from other organisms. RIL is selected for by resistance to chloramphenicol (30 μg/ml). The presence of the plasmid can improve production of recombinant proteins.

The *E. coli* strain ER2738 (F' proA+B+ lacIq Δ(lacZ)M15 zzf::Tn10 (TetR) fhuA2 Δ(lac-proAB) glnV thi-1 Δ(hsdS-mcrB)5) is a robust F+ strain with a rapid growth rate and is particularly well-suited for M13 propagation.

Yeast Strains

The prototrophic *K. lactis* strain GG799 (Matα [pGKl1^{+}]) is a wild type industrial isolate (DSM Food Specialities, Delft, The Netherlands) that grows to very high cell density and efficiently secretes heterologous proteins.

Protein expression and purification

The following protocols describe the general method. Any modifications used for a particular protein are described in the previous chapters and summarized in Table 2. Parameters such as OD and temperature of induction, as well as IPTG concentration and length of induction for the expression were evaluated to optimize expression. For purification, varying concentrations of imidazole were employed in the case of His-tagged proteins.

Production of His-tagged recombinant protein in E. coli

- Induction of expression

A pre-culture was prepared the night before induction by inoculating 10 ml of LB + ampicillin (and chloramphenicol if RIL strain used) with a fresh colony and incubated at 37°C overnight. 1 l of LB + amp was inoculated with 2 ml of pre-culture and grown to an OD_{600}= 0.5-0.6. IPTG was then added. Induction was performed at 37°C or 15°C for period of time ranging from 3 hrs to overnight. The cells were harvested by centrifugation, 15 min at 3500 rpm. The pellet was frozen at -20°C until further use.

- Cell lysis

The cell pellet from a 1 l culture was thawed and resuspended in 100 ml lysis buffer (40 mM phosphate pH8, 300 mM NaCl, 10 mM Imidazole) and sonicated in a metal beaker for 10 min (5x2 min, with 2 min stay between each sonication, 50 % duty, pulse). The solution was then clarified by centrifugation, 20 min at 12,000 rpm at 4°C, and the supernatant processed for purification.

- Protein purification

A volume (10 ml) of resin was washed with 100 ml of lysis buffer and the column was packed. The clarified lysate was loaded on the column and the flow through was collected at a speed of 1 ml/ml. The column was first washed with 100 ml of the lysis buffer (wash 1), then 100 ml of the wash buffer (40 mM phosphate pH8, 300 mM NaCl,

20 mM Imidazole, wash 2). The bound protein was eluted in 5 ml fractions with an elution buffer (40 mM phosphate pH8, 300 mM NaCl, 50-250 mM Imidazole). The fractions were analyzed for purity, concentration and activity.

- Analysis of the purification

SDS-PAGE was used to analyze the purification. Samples collected during the procedure (sonication, supernatant, pellet, flow through, washes and elutions) were run on a 4-20% or 12-20% Tris-Glycine gel. The samples were mixed with 3X loading buffer (187.5 mM Tris-HCl pH 6.8, 6% (w/v) SDS, 30% glycerol and 0.03% (w/v) phenol red, 125 mM DTT) and run in a Tris-Glycine running buffer (25 mM Tris Base, 192 mM Glycine, 0.1% SDS pH 8.3) at 35 mA per gel.

Enzyme	Strain or medium	His-tag position (Terminus)	[IPTG] mM	T° induction	Length of induction	[Imidazole] mM
B. malayi iPGM	ER2566	C-terminal	100	37°C	3 h	60
C. elegans iPGM	ER2566	C-terminal	100	15°C	overnight	60
E. coli dPGM	ER2566	C-terminal	300	37°C	3 h	200
E. coli dPGM	ER2566	C-terminal	300	37°C	3 h	100
H. sapiens dPGM	ER2566	C-terminal	300	37°C	3 h	100
O. volvulus iPGM	ER2566	C-terminal	100	37°C	3 h	50
T. brucei iPGM	LB + 1 M sorbitol+ 2.5 mM betaine	N-terminal	1	30°C	overnight	100
Wolbachia PPDK	RIL strain	C-terminal	100	15 °C	overnight	50

Table 2: Optimal conditions for expression and purification of various His-tagged proteins.

Production of recombinant Trypanosoma brucei iPGM (Tb-iPGM) in E. coli using the impact system

- Expression

The optimal conditions for expression of the Tb-iPGM using the IMPACT was similar to that described for the His-tagged protein.

- Cell lysis

The cell pellet from 1 l culture was thawed and resuspended in 100 ml lysis buffer (20 mM Tris-HCl pH7, 500 mM NaCl)) and sonicated in a metal beaker for 10 min (5x2 min, with 2 min rest between each sonication, 50 % duty, pulse). The solution was then clarified by centrifugation 20 min, at 12,000 rpm at 4°C, and the supernatant processed for purification.

- Protein purification

A volume (20 ml) of resin was washed with 200 ml of lysis buffer and the column was packed. The clarified lysate was loaded on the column and the flow through was collected at a maximum speed of 1 ml/ml. The column was washed with 400 ml of the column buffer (20 mM Tris-HCl pH7, 500 mM NaCl, wash), to induce the cleavage of the intein, the resin was flushed with 60 ml of cleavage buffer (20 mM Tris-HCl pH 8.5, 500 mM NaCl, 40 mM DTT) and incubated overnight at 4°C. The bound protein was eluted in 5 ml fractions. The fractions were analyzed for purity, concentration and activity. Glycerol was added to a final concentration of 50% and the solution stored at -20°C.

Synthèse en Français

Introduction

Maladies négligées et parasites filaires

La majorité des maladies négligées chez l'humain sont causées par des parasites. Elles sont principalement trouvées dans les pays tropicaux et touchent plus d'un milliard de personnes, souvent dans les régions les plus pauvres du monde et ces maladies sont en fait une grande cause de pauvreté. Certaines de ces infections sont mortelles mais la plupart sont souvent handicapantes, défigurantes et stigmatisantes. Dans la plupart des cas, les possibilités de traitements sont inadéquates ou n'existent pas et le marché potentiel pour les compagnies pharmaceutiques n'est pas suffisant pour les attirer. Quelques exemples de maladies négligées causées par des parasites protozoaires sont la malaria, la leishmaniose et les trypanosomiases (maladie de Chagas et maladie du sommeil). Les vers parasitaires (helminthes) sont responsable des Bilharzioses et des filarioses (lymphatique et onchocercose). L'Organisation mondiale de la santé (OMS) et le Programme spécial de recherche et de développement concernant les maladies tropicales (TDR) ont toujours été concernés par les maladies infectieuses dans les pays en voie de développement afin d'en améliorer la prévention, la détection et les traitements. Depuis plus de 25 ans, New England Biolabs a effectué des recherches sur les filarioses humaines. Dr Donald Comb, fondateur de NEB, a reconnu l'importance de la recherche fondamentale sur ces maladies négligées pour découvrir de nouvelles méthodes de contrôle des parasites et ainsi améliorer la qualité de vie dans les régions où les infections filaires existent.

Les filarioses sont un groupe de maladies humaines et animales causées par des nématodes parasitaires filiformes ou filaires de l'Ordre des Spirurida. Le mâle et la femelle adultes vivent dans les vaisseaux, cavités corporelles ou les tissus de l'hôte vertébré (hôte définitif) et sont vivipares, produisant de jeunes microfilaires (premier stade larvaire) qui circulent dans le sang ou la peau. Les microfilaires sont ingérés par des insectes piqueurs-suceurs (hôte intermédiaire) dans lesquels ils évoluent et se transforment pour atteindre une forme infectante (troisième stade larvaire) avant d'être

transmis à un nouvel hôte définitif. Les filarioses lymphatiques et l'onchocercose sont les maladies les plus sérieuses de ce groupe.

Filariose lymphatique

Les filarioses lymphatiques sont causées par des filaires qui vivent dans le système lymphatique de l'hôte humain. Plusieurs espèces responsables de la maladie ont été identifiées et incluent *Wuchereria bancrofti* (*W. bancrofti*)*, Brugia malayi* (*B. malayi*) et *Brugia timori* (*B. timori*).

Les parasites de la filariose lymphatique sont transmis à l'humain par une grande variété de moustiques (*Anopheles*, *Culex*, *Aedes*, *Mansonia*). *W. bancrofti* est de loin le plus prévalent, étant responsable d'environ 90% des cas dans les zone tropicales et sub-tropicales du monde. *B. malayi* est confiné au sud et sud-est de l'Asie tandis que *B. timori* est uniquement trouvé au Timor et les îles avoisinantes. Les filarioses lymphatiques sont après la malaria, la seconde plus importante/prévalente maladie due à un vecteur dans le monde avec plus de 120 millions de personnes atteintes et 1,3 milliard à risque (Melrose, 2002).

Les filarioses lymphatiques sont considérées comme une des maladies les plus défigurantes et handicapantes (Melrose, 2002 ; Leggat *et al.*, 2004), ceci étant largement du au gonflement des membres inférieurs ou éléphantiasis ainsi que les atteintes des parties génitales dans le cas de l'hydrocèle. Le génome de *B. malayi* a récemment été séquencé (Ghedin *et al.*, 2007) représentant le premier génome de parasite helminthique à être complété, et a fourni une grande quantité d'informations pour les études sur les filaires.

La morphologie des vers de *B. malayi* est très similaire et celle de *W. bancrofti*. Les deux espèces appartiennent à la clade III de l'Ordre des Spirurida (Figure 1) (Dorris *et al.*, 1999).

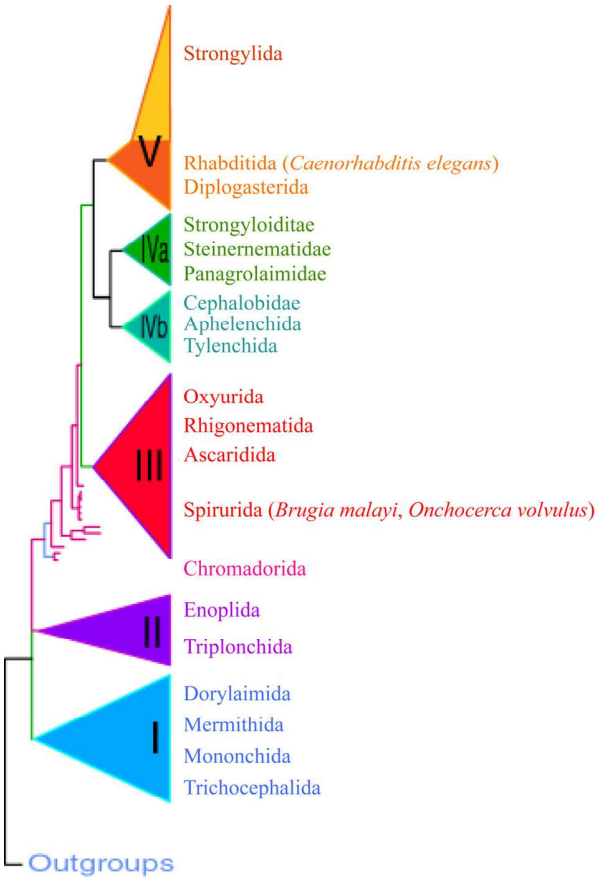

Figure 1: La structure phylogénétique des Nematoda révélée par l'analyse des séquences complètes des ADNr de la petite sous-unité. (adapté de (Dorris *et al.*, 1999))

Les femelles adultes sont plus grandes que les mâles, pouvant mesurer jusqu'à 43.5-55.0 x 0.13-0.17 mm, tandis que les mâles font 13.5-23.3 x 0.07-0.08 mm (Sasa, 1976). Ils peuvent vivre plus de 10 ans dans l'hôte, période durant laquelle la femelle relâche en permanence des microfilaires. Ceux ci ont une durée de vie de plusieurs mois et ont une périodicité nocturne, ce qui signifie qu'ils circulent dans le sang durant la nuit et se réfugient dans les tissus le jour. Les moustiques vecteurs les plus communs pour la filariose de Malaisie font partie de l'espèce *Mansonia*, piqueuse nocturne. Après avoir été ingérés par le vecteur, le parasite se développe et subi 2 mues sur une période d'une dizaine de jours pour atteindre le stade L3 infectieux, capable de transmettre l'infection à un autre humain (Muller and Baker, 1990). Chez l'humain, 2 autres mues sont subies afin d'atteindre le stade adulte qui se reproduira et produira des microfilaires (Figure 2).

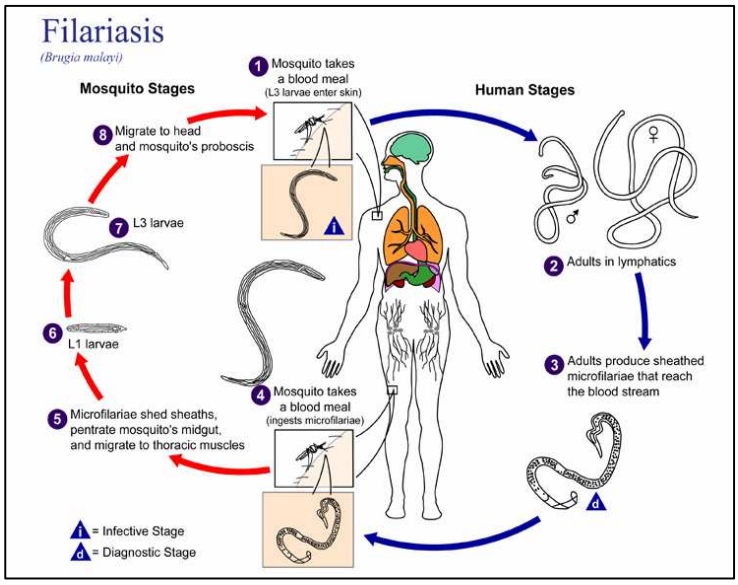

Figure 2: Cycle de vie de la filaire *B. malayi*.
(Source: http://www.dpd.cdc.gov/dpdx)

Le diagnostic de la filariose lymphatique en absence de gonflements est un problème et la méthode la plus commune utilisée sur le terrain est la détection de microfilaires dans le sang prélevé la nuit, et détermination de l'espèce responsable par coloration avec le Giemsa (Figure 3). Plus récemment des techniques d'amplification en chaîne par polymérase (ACP), la détection d'antigènes, les rayons X et l'ultrasonographie ont été utilisés dans les laboratoires possédant plus d'équipement dans les pays endémiques (Leggat *et al.*, 2004).

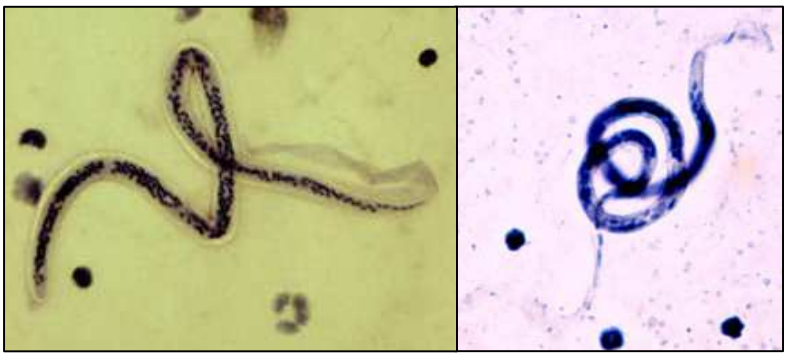

Figure 3: Les agents responsables des filarioses lymphatiques.
Frottis sanguins contenant des microfilaires de *Wuchereria bancrofti* colorés à l' hematoxyline (panneau de gauche) et de *Brugia malayi* colorés au Giemsa (panneau de droite). (Source: wikipedia.org)

Onchocercose

Au jour d'aujourd'hui, environ 18-40 millions de personnes sont atteintes d'onchocercose ou cécité des rivières. La maladie est endémique dans plus de 36 pays en Afrique, partiellement dans la péninsule Arabe et en Amérique centrale et du sud (OMS, 2006). C'est la deuxième cause de cécité infectieuse dans le monde, pesant un grand poids sur un plan économique en raison des invalidités qu'elle cause (Burnham, 1998; Hoerauf *et al.*, 2003).

Le parasite responsable est la filaire *Onchocerca volvulus* (*O. volvulus*), transmise par des petites mouches noires du genre *Simulium*. Ces mouches sont confinées dans les rapides des rivières pour leur développement d'où le nom de cécité des rivières. *O. volvulus* appartient également à la clade III et l'Ordre des Spirurida (Figure 1) (Dorris *et al.*,1999) et partage un certain nombre de caractéristiques avec les parasites des filarioses lymphatiques.

Les adultes ont une durée de vie de 12-15 ans et la femelle est vivipare, mesurant jusqu'à 400 x 0.3 mm, tandis que le mâle est plus petit, atteignant 30 x 0.2 mm (Muller and Baker, 1990). Ils vivent dans les tissus sous cutanés sur le torse, la tête et près des jointures où ils peuvent former des kystes onchocerquiens. Les microfilaires circulent dans la peau et les yeux et mesurent 280-330 x 6-9 μm (Muller and Baker, 1990). Après avoir été ingérés par la *simulie*, les microfilaires muent deux fois pour devenir infectieux et atteignent une taille de 440-700 x 19 μm (Figure 4). Les infections par *O. volvulus* sont caractérisées par une peau écailleuse, irritée et noduleuse ainsi que par la cécité dans les pires des cas. Les microfilaires causent la cécité en raison d'une réaction inflammatoire lors de leur mort dans la cornée.

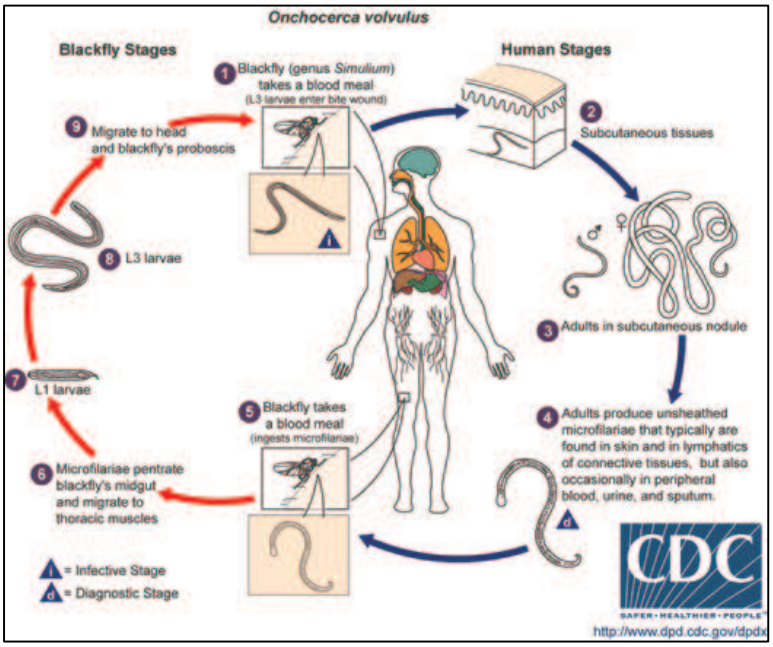

Figure 4: Cycle de vie de la filaire *O. volvulus*.
(Source: http://www.dpd.cdc.gov/dpdx)

Le diagnostic des infections par *O. volvulus* s'effectue par palpations des nodules et par détection des microfilaires dans l'œil. La méthode la plus commune et la plus douloureuse se fait par isolement des microfilaires à partir de biopsies cutanées exsangues après incubation de celles-ci dans une solution saline pour 30 min. Les parasites, très mobiles, qui émergent de ces biopsies peuvent être visualisés sous microscope. Un test de Mazzotti peut être effectué dans certains cas en utilisant une dose de 50 mg de diéthylcarbamazine qui va provoquer des flambées d'urticaires et des érythèmes par réaction allergique due à la lyse massive des microfilaires. Plusieurs tests immunologiques et par amplification en chaine par polymérase sont également utilisés dans les laboratoires plus développés (Enk, 2006).

Traitements

Le nombre de médicaments disponibles pour traiter les filarioses humaines est incroyablement faible. Trois substances sont actuellement utilisées, l'ivermectine (IVM), l'albendazole (ABZ) et le diéthylcarbamazine (DEC) et ont été découvertes dans le domaine vétérinaire et ont ensuite été adaptées pour la santé humaine. Depuis 30 ans, l'OMS et TDR ainsi que des agences gouvernementales ont collaborés avec les compagnies pharmaceutiques pour essayer de contrôler et éliminer ces maladies à l'aide des campagne d'administration en masse des médicaments (MDA) (Woods *et al.*, 2007) et par le contrôle des vecteurs. Merck and Co Inc. a été la première compagnie à participer dans les années 80 et a généreusement fourni le Mectizan (IVM) à divers pays d'Afrique pour l'élimination de l'onchocercose. Plus récemment, GlaxoSmithKline a joint l'effort avec la donation d'albendazole. Ces médicaments sont distribués lors les campagnes annuelles d'administration de masse, soit seul (IVM) pour l'onchocercose ou en association pour les filarioses lymphatiques (ABZ plus IVM ou IVM plus DEC dans les régions où l'onchocercose n'est pas présente) (Townson *et al.*, 2007).

Ces trois médicaments ciblent les stades immatures des filaires (microfilaires et stades larvaires) et de longs traitements sont requis pour couvrir la période reproductive des vers adultes (jusqu'à 15 ans pour *O. volvulus*) afin d'interrompre la transmission. Les limitations principales de ces traitements sont les effets limités sur les adultes et l'apparition de résistance. Les résistances ont été mises en évidence depuis un certain temps chez les animaux traités pour les infections à nématodes intestinaux après l'utilisation intense et répandue des mêmes classes médicamenteuses (Waghorn, 2006). La résistance à l'ivermectine a été récemment documentée dans des populations infectées par *O. volvulus* (Prichard, 2007). Par conséquent, il y a un besoin urgent d'identifier de nouvelles cibles thérapeutiques et développer de nouvelles thérapies pour les infections filaires humaines.

Wolbachia en tant que cible thérapeutique

Depuis quelques années, les symbiotes intracellulaires obligatoires de type α-protéobactériens du genre *Wolbachia*, qui sont présents dans la plupart des filaires (Kozek and Marroquin, 1977), ont été étudiés comme une nouvelle approche de chimiothérapie. Ils sont trouvés chez les mâles comme les femelles ainsi qu'à tous les stades larvaires. Leur présence dans les oocytes, les œufs et les microfilaires (McLaren e*t al.*, 1975; Kozek and Marroquin, 1977) indiquent que la bactérie est maintenue dans la population par transfert vertical. Elles sont majoritairement localisées dans les cordes latérales et les oocytes (Hoerauf and Pfarr, 2007). Ces bactéries sont essentielles pour le développement des vers, leur fertilité et survie (Hoerauf et *al.*, 2000; Taylor *et al.*, 2005; Hoerauf and Pfarr, 2007; Pfarr and Hoerauf, 2007). Des antibiotiques (principalement de la famille des tétracyclines) ont été montrés efficaces contre *Wolbachia* et ces traitements ont résulté dans la mort des filaires (Taylor *et al.*, 2005; Hoerauf, 2006). Néanmoins, les fortes doses et la longueur du traitement nécessaires pour avoir cet effet excluent pour le moment l'utilisation d'antibiotiques pour le contrôle des filarioses. Cependant, ces études démontrent la faisabilité du développement de traitements visant le symbiote *Wolbachia* (Hoerauf et *al.*, 2000; Pfarr and Hoerauf, 2006).

Le Processus de découverte de médicament

Comme mentionné précédemment, les médicaments utilisés couramment pour le traitement des filarioses humaines ont été découverts dans l'industrie vétérinaire. Le processus de découverte implique largement le criblage de collections de composés chimiques pour une activité contre les parasites intactes, soit en culture, soit dans des animaux infectés. Cette approche requiert de grande quantité de matériel parasitaire et de composés chimiques. La récente disponibilité d'informations génomiques et l'amélioration des méthodes de criblage à haut débit ont permis aux scientifiques d'affiner leur façon de chercher de nouveaux médicaments (Woods *et al.*, 2007; Kaminsky *et al.*, 2008). Plusieurs possibilités sont maintenant disponibles comme il a été décrit (Hudson and Nwaka, 2007). Celles ci incluent : *la sélection de composés sur bases*

biologiques où des composés « actifs » de compagnies ayant des programmes insecticides et anti-helminthiques vétérinaires/agrochimiques sont évalués, ce criblage peut être effectué avec *Caenorhabditis elegans* (nématode modèle) qui est adaptable au criblage à haut débit ;et *la sélection de composés sur bases biochimiques* ou des familles de composés issues de programmes pharmaceutiques non parasitaires sont testés sur des vers entiers ou dans un criblage à haut débit sur des cibles parasitaires spécifiques.

Utilisation de la génomique et de données fonctionnelles (C. elegans) pour identifier de nouvelles cibles thérapeutiques

Le séquençage des génomes de *B. malayi* (Ghedin *et al.*, 2007) et de son symbiote *Wolbachia* (*w*Bm) (Foster *et al.*, 2005) récemment complétés ont fournis une formidable opportunité pour la découverte de nouvelles cibles thérapeutiques. Cependant, la validation de ces cibles thérapeutiques est un défi car les techniques de génomique fonctionnelle sont limitées (tel que l'interférence ARN (iARN) dans les filaires) ou inexistantes (pour le *Wolbachia* filaire) dans ces systèmes. Pour contourner ce problème, notre laboratoire a utilisé le nématode apparenté *C. elegans* en tant que substitut pour *B. malayi*. *C. elegans* a été proposé à plusieurs occasions en tant que bon modèle pour les autres nématodes (Aboobaker and Blaxter, 2000; Britton and Murray, 2006; Kaletta and Hengartner, 2006; Holden-Dye and Walker, 2007). *C. elegans* appartient à la clade V et l'Ordre des Rhabditida (Figure 1). Le génome de *C. elegans* est complètement séquencé et considérablement annoté. Plusieurs études de iARN sur le génome entier ont été effectuées et ces données sont disponibles publiquement dans Wormbase (Bieri *et al.*, 2007). Notre laboratoire a utilisé ces informations pour développer une méthode de sélection sur le génome entier afin identifier de nouvelles cibles thérapeutiques (Foster *et al.*, 2005; Kumar *et al.*, 2007).

La phosphoglycérate mutase indépendante en tant que potentielle cible thérapeutique chez les filaires et Wolbachia

Une cible particulièrement intéressante identifiée en utilisant cette approche est l'enzyme phosphoglycérate mutase indépendante (PGMi). Les PGMs catalysent la

conversion réversible du 2- et 3-phosphoglycérate dans la glycolyse et la néoglucogenèse. Bien que ces voies métaboliques soient très conservées parmi différents organismes, deux formes distinctes de PGM existent, PGMi et la phosphoglycérate mutase dépendante d'un cofacteur (PGMd). Les mammifères, en plus de certaines bactéries et plantes possèdent uniquement la PGMd tandis que d'autres organismes ont uniquement la PGMi ou les deux (Carreras *et al.*, 1982; Fothergill-Gilmore and Watson, 1990; Fraser *et al.*, 1999). Il n'y a pas de similarité de séquences entre les deux enzymes et elles opèrent par des mécanismes différents (Jedrzejas *et al.*, 2000; Bond *et al.*, 2001).

PGMi appartient à la superfamille des phosphatases alcalines. Elle catalyse le transfert du phosphate grâce à un résidu serine et elle n'est pas sensible au vanadate (Britton *et al.*, 1971; Jedrzejas and Setlow, 2001; Jedrzejas, 2002; Rigden *et al.*, 2003). PGMi a également été identifiée comme cible thérapeutique dans un certain nombre de pathogènes, parmi lesquels les parasites protozoaires *Trypanosoma brucei* (Chevalier *et al.*, 2000; Collet *et al.*, 2001) et *Leishmania mexicana* (Guerra *et al.*, 2004) où elle a été poursuivie en tant que cible thérapeutique.

PGMd appartient à la superfamille des phosphatases acides (Jedrzejas, 2000). Cette enzyme requiert le cofacteur 2,3 bisphosphoglycérate pour transférer le phosphate entre le substrat et le produit grâce à un intermédiaire phospho-histidine (Rigden *et al.*, 2002; Rigden *et al.*, 2003). Elle est sensible au vanadate (Carreras *et al.*, 1980).

Des études précédemment réalisées dans notre laboratoire ont montré que les nématodes possèdent uniquement la PGMi et que celle ci a un rôle essentiel dans le développement de *C. elegans*, indiquant son potentiel en tant que cible thérapeutique chez les filaires (Zhang *et al.*, 2004). Notre intérêt à développer PGMi en tant que cible thérapeutique nous a conduits à une étude collaborative avec le Dr. Najib El-Sayed (George Washington University, anciennement à TIGR) sur le parasite *Trypanosoma brucei*, agent causal de la maladie du sommeil ou trypanosomiasis africaine chez l'humain.

La phosphoglycérate mutase cofacteur indépendante en tant que cible thérapeutique chez les trypanosomes africains.

Deux parasites de la famille des Trypanosomatidae sont responsables de la maladie. La forme chronique est causée par *Trypanosoma brucei gambiense* en Afrique de l'Ouest, tandis que *T. b. rhodensiense* cause la forme aigue et plus sévère trouvée en Afrique de l'Est et Centrale. Les deux parasites sont transmis par la mouche TséTsé (*Glossina* spp). Communément, après infection, le patient développe une réaction cutanée appelée chancre d'inoculation. Le parasite envahit ensuite le système nerveux central (phase neuro-méningée), conduisant à une encéphalopathie chronique avec dégradation générale des fonctions cognitives jusqu'à ce que le patient entre en phase terminale de somnolence (Barrett *et al.*, 2003; Croft *et al.*, 2005). *T. brucei* sont des parasites protistes unicellulaires de la classe des Kinetoplastids.

Le diagnostic de la maladie du sommeil est normalement fait par identification des parasites dans le sang, le liquide cérébrospinal ou des aspirats de nodules lymphatiques. Des tests ELISA et d'agglutination peuvent aussi être utilisés (Muller and Baker, 1990). Un certains nombre de médicaments peuvent être prescrits pour le traitement de la maladie, pourtant, des problèmes de toxicité et d'inefficacité dans les phases neuro-méningée existent et des résistances apparaissent (Croft *et al.*, 2005; Delespaux and de Koning, 2007). La suramine et la pentamidine sont utilisées dans les stades précoces de la maladie. Trois autres médicaments sont disponibles pour le traitement des patients au stade avancé, le melarsoprol dérivé de l'arsenic, qui a énormément d'effets toxiques, l'eflornithine (α-difluoromethylornithine or DFMO), un dérivé du melarsoprol moins toxique et enfin le nifurtimox, utilisé en association avec le melarsoprol.

Analyse génomique de la glycolyse chez les nématodes et Wolbachia

La découverte de PGMi en tant que potentielle cible thérapeutique chez les nématodes et *w*Bm nous a conduits à effectuer une analyse du génome plus poussée pour les voies glycolytique et néoglucogénique de ces organismes. La glycolyse consiste en la transformation du glucose en pyruvate et se déroule selon la voie d'Embden-Meyerhof. Chez la plupart des eucaryotes et procaryotes, celle-ci se déroule dans le cytoplasme mais

la situation est un peu différente chez les plantes et les protozoaires ou certaines enzymes de la glycolyse ont été trouvées dans les chloroplastes (plantes) et les glycosomes (*T. brucei*). La glycolyse standard se déroule en 10 étapes (Figure 5) ou les étapes 1,3 et 10 sont irréversibles lorsque catalysées par les enzymes standards (Stryer, 1995). La glycolyse résulte dans la plupart des cas dans un gain de 2 ATP et la production de NADH, fournissant ainsi de l'énergie à la cellule. Le pyruvate formé peut ensuite entrer le cycle de Krebs ou être fermenté. Cinq des 10 enzymes de la glycolyse ont des alternatives (Figure 5) et la distribution de celles-ci dans les organismes étudiés dans ce travail doctoral est présentée dans le (Tableau 1). Les mammifères et *Escherichia coli* sont inclus pour comparaison.

Notre analyse a révélé que le symbiote intracellulaire *Wolbachia* ne possède pas la pyruvate kinase, mais semble au contraire utiliser la pyruvate phosphate dikinase qui pourrait potentiellement catalyser la réaction dans la voie glycolytique et néoglucogénique. Il est intéressant de noter que la glycolyse chez *Wolbachia* semble débuter au fructose 1,6 bisphosphate tandis que la présence de la fructose 1,6 bisphosphatase indique que la néoglucogenèse se terminerait avec le fructose 6 phosphate (Foster *et al.*, 2005a).

Enzymes Standard/ alternative	*Mammifères*	*E. coli*	*T. brucei*	*C. elegans*	*B. malayi*	*O. volvulus*	*w*Bm
HEX / GLK	Les deux	GLK	HEX	HEX	HEX	Pas de données	Absente
GPI	présente	présente	présente	présente	présente	présente	Absente
PFK / PFP	PFK	PFK	PFK	PFK	PFK	PFK	Absente
FBA I / FBA II	FBA I	FBA I	FBA I	FBA I	Pas de données	FBA I	FBA I
TPI	présente	présente	présente	présente	présente	présente	présente
GAP	présente	présente	présente	présente	présente	présente	présente
PGK	présente	présente	présente	présente	présente	présente	présente
dPGM / iPGM	PGMd	Les deux	PGMi	PGMi	PGMi	PGMi	PGMi
ENO	présente	présente	présente	présente	présente	présente	présente
PK / PPDK	PK	PPDK	Les deux	PK	PK	PK	PPDK

Tableau 1: Distribution des enzymes glycolytiques standards et alternatives dans les organismes étudiés.

Les enzymes présentées dans le tableau sont HEK/GLK (héxokinase/glucokinase), GPI (glucose-6-phosphate-isomerase), PFK/PFP (6-phosphofructokinase/pyrophosphate::fructose 6-phosphate phosphotransférase), FBA I/BFA II (fructose-bisphosphate aldolase Classe I/classe II), TPI (triose phosphate isomérase), GAP (glycéraldehyde 3-phosphate déshydrogénase), PGK (Phosphoglycérokinase) PGMd/PGMi (phosphoglycérate mutase cofactor-dépendante/ cofactor-indépendante), ENO (énolase) et PK/PPDK (pyruvate kinase/pyruvate phosphate dikinase).

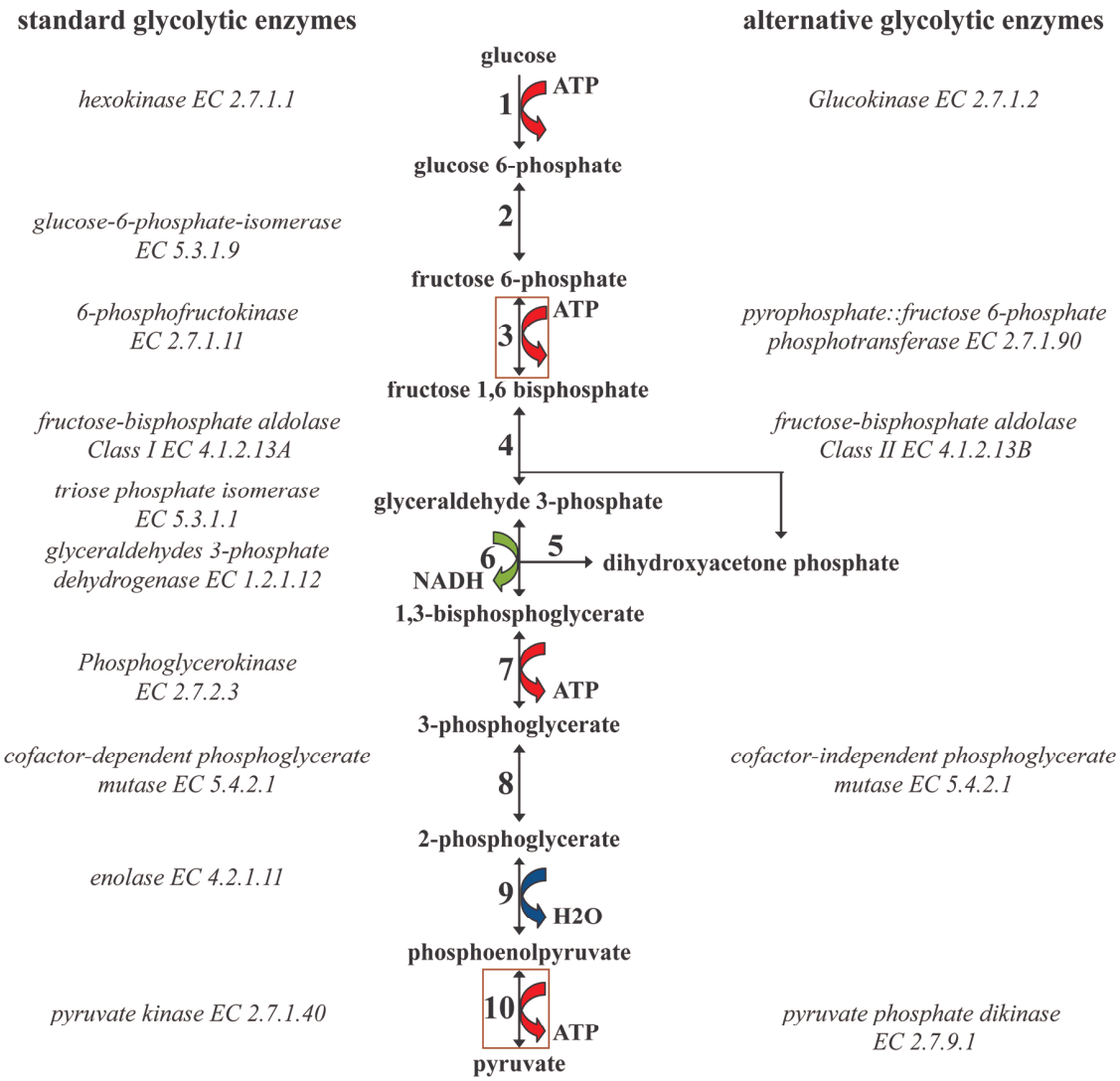

Figure 5: La voie glycolytique utilisant les enzymes standards et alternatives.

Les cadres indiquent les réactions (3 et 10) qui sont réversibles quand catalysées par les enzymes alternatives.

La pyruvate phosphate dikinase en tant que cible thérapeutique chez Wolbachia

La PPDK est une protéine qui catalyse la conversion réversible du phosphoenolpyruvate, de l'AMP et du PPi en pyruvate, ATP et Pi. (Wood *et al.*, 1977). La PPDK est une alternative permettant de conserver de l'énergie en comparaison avec la pyruvate kinase, car elle permet la production de 4 ATP par molécule de glucose (Mertens, 1993). La plupart des organismes, y compris les mammifères possèdent

uniquement la pyruvate kinase. Par conséquent, l'absence de PPDK chez les mammifères la rend intéressante comme cible thérapeutique chez *Wolbachia*. Comme il n'y a pas d'homologie de séquence entre la PPDK et la PK, il est possible que des inhibiteurs spécifiques de la PPDK puissent être identifiés. La PPDK a également été trouvée chez les parasites protozoaires et son rôle important chez *Entamoeba histolytica* et *Giardia lamblia* (Saavedra-Lira and Perez-Montfort, 1996; Feng *et al.*, 2008) ont stimulé des intérêt considérables dans cette enzyme comme une cible potentielle pour le développement de nouvelles thérapies anti-protozoaires.

Buts de l'étude

Un des buts de mon travail doctoral a été d'étudier les PGMis de nématodes plus en détail pour déterminer si les enzymes de filaires et de *C. elegans* avaient des caractéristiques biochimiques similaires, ce qui indiquerait qu'un inhibiteur unique serait probablement actif et efficace contre toutes les enzymes de nématodes (Chapitre 1). Cette information indiquerait également la validité d'utiliser *C. elegans in vivo* pour des cribles afin d'évaluer l'efficacité d'inhibiteurs enzymatiques. Il est intéressant de noter que notre analyse génomique a indiqué que cette enzyme pourrait être présente chez le symbiote *Wolbachia*. Nous avons ainsi été intéressé dans le clonage et l'expression de la PGMi putative pour déterminer si elle pourrait également être une cible thérapeutique (Chapitre 2). Notre intérêt à développer PGMi en tant que cible thérapeutique nous a conduit à une étude collaborative avec le Dr. Najib El-Sayed (George Washington University, anciennement a TIGR) sur le parasite *Trypanosoma brucei*, agent causatif de la maladie du sommeil ou trypanosomiasis africaine chez l'humain (Chapitre 3).

D'après notre analyse génomique de la glycolyse de *Wolbachia* ; nous avons proposé que la PPDK pourrait être une bonne cible thérapeutique, nous avons donc par conséquent été intéressés par le clonage et l'expression de la PPDK prédite de *Wolbachia* (*w*Bm-PPDK) afin d'avoir un aperçu du métabolisme énergétique de ce symbiote et pour développer cette enzyme en tant que cible thérapeutique (Chapitre 4).

Le but ultime de nos études sur la PGMi et la PPDK est la découverte

d'inhibiteurs spécifiques qui peuvent représenter des composés meneurs pour de plus amples développements comme composés anti-parasitaire. J'ai donc criblé plusieurs banques de phages exprimant des peptides en surface pour tenter de trouver des inhibiteurs de la PGMi (Chapitre 5). J ai également effectué des études biochimiques sur les enzymes PGMd et PGMi d'*E. coli* en parallèle au développement de souches mutantes qui ont été construites pour une utilisation lors du criblage sur cellule pour étudier la spécificité d'inhibiteurs de la PGMi et étudier les rôles respectifs de ces enzymes chez *E. coli* (Chapitre 6).

Discussion et Conclusions

La première étude présentée ici décrit le clonage et l'expression de la PGMi d'*O. volvulus* ainsi que la caractérisation biochimique détaillée des trois PGMis de nématodes d'*O. volvulus*, de *B. malayi* et de *C. elegans*. Nous avons montré que les trois enzymes sont très conservées, entre elles d'une part (>70%) mais également avec les PGMi précédemment décrites. Comme les autres PGMis, les enzymes de nématodes ont été démontrées dépendante d'un ion métallique. Nous avons déterminé que les ions magnésium et manganèse étaient les ions préférés, en contraste avec l'enzyme bien caractérisée du parasite protozoaire *T. brucei* qui préfère les ions cobalt (Collet *et al.*, 2001). De plus amples caractérisations ont révélé que les trois enzymes de nématodes possèdent les qualités classiques des PGMis, en étant insensibles au vanadate et n'étant pas activées par le 2,3 bisphosphoglycérate, comme c'est le cas pour les PGMds. Les résultats de cette étude indiquent que les enzymes de nématodes partagent des caractéristiques enzymatiques et cela indique qu'un inhibiteur unique serait probablement efficace contre toutes ces enzymes. La grande similarité entre les enzymes de filaires et celle de *C. elegans* valide l'utilisation de ce dernier dans des criblages sur organisme entier pour évaluer l'efficacité d'inhibiteurs anti-nématodes.

Un certain nombre de filaires, y compris ceux responsable des filarioses lymphatiques et de l'onchocercose sont infectés par un symbiote intracellulaire bactérien, *Wolbachia*. Cette bactérie est un symbiote mutualiste et est considérée comme une cible

thérapeutique (Hoerauf and Pfarr, 2007; Taylor *et al.*, 2005) car leur élimination grâce à des antibiotiques (principalement de la famille des tétracyclines) conduit à la mort de leur hôte (Hoerauf *et al.*, 2001; Hoerauf *et al.*, 2003a; Hoerauf *et al.*, 2003b). Le génome de *Wolbachia*, symbiote de *B. malayi* est récemment devenu disponible (Foster *et al.*, 2005). L'analyse génomique de cet organisme qui nous a conduits à découvrir la présence d'une PGMi putative. La deuxième étude présentée ici, décrit le clonage et l'expression de cette PGMi putative. *w*Bm-PGMi Recombinante a été un réel problème à produire en raison de son faible niveau d'expression et de solubilité ainsi que son manque d'activité. Plusieurs systèmes d'expression ont été explorés pour l'expression bactérienne (*E. coli*), ainsi que diverses souches de *E. coli*, sans succès. Les problèmes d'expression ont été résolus en utilisant un système d'expression dans *K. lactis*, qui nous a permis de produire et d'évaluer l'activité de l'enzyme recombinante. Nos résultats démontrent que le symbiote *Wolbachia* possède également une PGMi active, ce qui justifie de plus amples recherches en tant que cible thérapeutique.

Nos études sur les PGMis de nématodes et *Wolbachia* ont débouché sur une collaboration avec un groupe travaillant sur le stade procyclique du parasite protozoaire *Trypanosoma brucei*. *T. brucei*, est le parasite responsable de la trypanosomiase humaine africaine ou maladie du sommeil, et de précédentes études ont révélé la présence de la PGMi dans cet organisme (Chevalier *et al.*, 2000). La troisième publication présentée décrit les résultats d'une étude sur le rôle de la PGMi au cours de la croissance de *T. brucei* procyclique *in vitro*. La séquence du génome prédit une PGMi codant une protéine avec une extrémité C-terminale différente de celle publiée précédemment (Chevalier *et al*. 2000). J'ai cloné et exprimé la protéine correspondante à la nouvelle séquence et montré que celle-ci effectivement encodait une PGMi active. Nous avons ensuite créé des lignées cellulaires du parasite avec une expression inductible de l'ARN double brin correspondant à la PGMi pour réduire le niveau de transcription du gène de la PGMi par iARN. J'ai mesuré les niveaux d'activité de la PGM dans des extraits cellulaires, et nous avons montré que la réduction de l'activité enzymatique corrélait avec une réduction des ARN messagers de la PGMi. Nous avons également montré que la croissance des cellules dépendait d'une expression normale de la PGMi prouvant ainsi

que cette enzyme est nécessaire pour la croissance et donc digne d'une étude plus poussée comme une cible thérapeutique dans le protozoaire *T. brucei*. Ces résultats sont en accord avec ceux obtenus par une autre équipe de travail sur la forme sanguine du parasite (Albert *et al.*, 2005).

Après avoir découvert la présence d'une PGMi active dans *Wolbachia* nous avons réalisé plus d'analyse génomique sur la glycolyse/néoglucogenèse de cet organisme. Nous étions particulièrement intéressés dans l'identification d'autres enzymes absentes chez les mammifères. La conversion du phosphoénolpyruvate en pyruvate dans la nature peut être catalysée par deux enzymes, à savoir la pyruvate kinase (PK), qui est l'enzyme présente dans la majorité des organismes, et la pyruvate phosphate dikinase (PPDK), qui est absente du génome des mammifères. Notre analyse a montré que *Wolbachia* ne possédait pas de PK mais encodait une PPDK putative. La quatrième étude décrit le clonage, l'expression et l'activité de la pyruvate phosphate dikinase putative de *Wolbachia.* L'expression et la purification de l'enzyme ont été difficile en raison de sa faible stabilité, cependant, après l'optimisation de l'expression et du protocole de purification, nous avons réussi à obtenir une grande quantité de protéine active et purifiée. Cette enzyme a été reportée comme une cible thérapeutique potentielle dans un petit nombre d'organismes parasitaires, à savoir *Entamoeba histolytica* et *Giardia lamblia*, ainsi que dans *T. brucei*. Fait intéressant, ces parasites possèdent à la fois PK et PPDK, et chez *T. brucei*, PPDK est localisée dans le glycosome, où elle aurait un rôle essentiel dans le métabolisme du pyruvate (Acosta *et al.*, 2004).

Nos études ont indiqué que PGMi et PPDK étaient des cibles thérapeutiques potentielles chez les filaires et/ou leur symbiote *Wolbachia*. Notre prochain objectif était d'identifier des inhibiteurs de ces enzymes. Nous avons choisi d'utiliser la technologie du « phage display » ou exposition sur phage, comme cette technique est disponible chez New England Biolabs. L'exposition sur phages est une méthode puissante pour l'identification de peptides qui se lient de façon spécifique à une protéine par l'analyse d'un grand nombre de peptides aléatoires exprimées à la surface d'un phage (Smith et Petrenko, 1997). La sélection des peptides se fait contre une cible et les phages élués peuvent ensuite être séquencés afin de relier la séquence nucléotidique au peptide

exprimé (Barbas *et al.*, 2001). Dans cette étude, j'ai criblé trois bibliothèques de phages exprimant des peptides aléatoires de 7 (linéaires ou cycliques) ou 12 acides aminés afin d'identifier des peptides se liant à la phosphoglycérate mutase indépendante d'*O. volvulus* purifiée. Plusieurs séquences de peptides consensus ont été obtenues mais malheureusement aucune activité inhibitrice n'a pu être détectée dans les conditions utilisées.

Nos études sur les PGMis de parasites/*Wolbachia* nous ont conduits à nous intéresser à la distribution des PGMis et PGMds dans la nature, et en particulier chez les pathogènes. Certains organismes possèdent la forme dépendante comme la levure et les mammifères (Carreras *et al.*, 1982; Fothergill-Gilmore and Watson, 1990), tandis que d'autres comme les nématodes possèdent la forme indépendante. Certaines bactéries comme *E. coli* encodent les deux formes de l'enzyme (Fraser *et al.*, 1999). Nous nous sommes donc intéressés aux les rôles respectifs de la PGMd et la PGMi chez *E. coli*. Dans la dernière étude, nous décrivons le clonage et l'expression de ces deux protéines d'*E. coli*, et évaluons leurs activités de PGMs, ainsi que les possibles activités phosphatases qui ont été proposées (Rigden et al., 2001). L'expression des deux enzymes a été réalisée avec succès et celles-ci se sont révélées actives dans notre test et n'ont pas montré d'activité phosphatase dans les conditions testées. La forme dépendante s'est avérée être inhibée par le vanadate. Nous avons ensuite créé des mutants déficients pour chaque enzyme et nous avons observé un défaut de croissance dans le mutant déficient en PGMd, causé par un retard dans la sortie de phase stationnaire. Ce défaut pouvait être corrigé par la surexpression de PGMd et PGMi. Les deux souches déficientes produites pourraient être utiles au criblage à haut débit d'inhibiteurs, et l'évaluation de leurs spécificités.

L'analyse des informations génomiques pour la découverte de nouvelles cibles thérapeutiques chez les filaires en est encore à ses balbutiements. Le travail présenté dans cette thèse démontre qu'une méthode de filtration génomique peut être utilisée pour l'identification de cibles thérapeutiques "pré-validées". Avec pour objectif le développement de la PGMi en tant que cible thérapeutique contre les filaires, j'ai produit un panel d'enzymes recombinantes comprenant des PGMis (*E. coli*, *T. brucei*, *C. elegans*,

B. malayi, *O. volvulus* et *Wolbachia*) et des PGMds (Humaine et *E. coli*) d'un certain nombre d'organismes. J'ai également produit des PPDK actives de *Wolbachia* et *T. brucei*. Ce groupe d'enzymes aidera à la recherche d'inhibiteurs enzymatiques ainsi que l'évaluation de leurs activités spécifiques. La Figure 6 montre une stratégie possible pour l'identification d'inhibiteurs de la PGMi utilisant ces enzymes recombinantes. La méthode peut être adaptée à n'importe quelle cible. La méthode d'expression sur phage qui a été utilisée pour identifier des peptides qui se lient à la PGMi d'*O. volvulus* illustre une approche pour identifier des inhibiteurs enzymatiques.

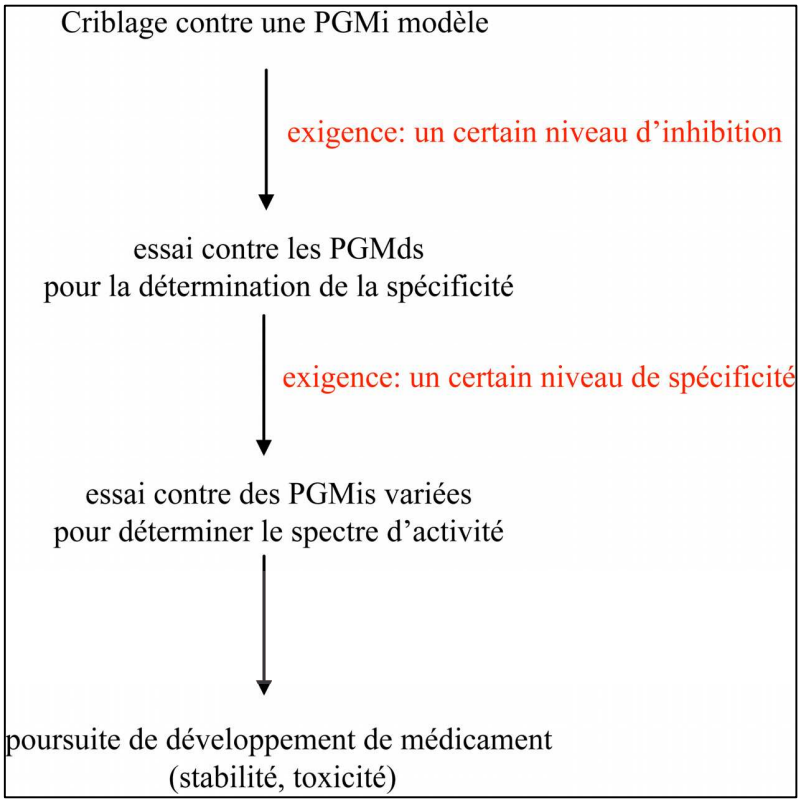

Figure 6 : Une voie de criblage pour la découverte d'inhibiteurs spécifiques de la PGMi.

Notre travail justifie d'avoir choisi la glycolyse comme cible pour la découverte de nouveaux médicaments pour traiter les filarioses. Fait intéressant, un des médicaments actuellement utilisés, à savoir l'albendazole, cible indirectement le métabolisme glucidique et la glycolyse. L'albendazole inhibe l'absorption du glucose par le biais de l'interruption du transport vésiculaire via les microtubules (Martin *et al.*,

1997), forçant le parasite à utiliser ses réserves de glycogène et aboutissant à sa mort. En outre, des analyses récentes de notre groupe (communication personnelle) de la Base de données des gènes essentiels (DEG) contenant des données provenant du criblage de 10 génomes bactériens pour les gènes essentiels (Zhang et Zhang, 2007), a révélé que les enzymes glycolytiques sont également essentielles chez un certain nombre de bactéries. Cela donne un certain soutien à l'exploration du métabolisme énergétique dans *Wolbachia* en tant que cible pour un effet thérapeutique.

Au cours de mon doctorat, j'ai développé des méthodes pour produire des enzymes recombinantes et actives des filaires et du *Wolbachia* filaire. Cela a impliqué une importante optimisation pour les protéines les plus difficiles à produire. Divers systèmes d'expression ont été examinés, ainsi que l'utilisation de différentes conditions d'expression et diverses souches. Les protocoles de purification ont également du être optimisés. Les informations disponibles dans la littérature sur l'expression de ces enzymes provenant d'autres organismes ont également été prises en considération et des spécialistes dans le domaine de l'expression de la protéine ont été consultés. Les connaissances acquises seront utiles dans la production d'autres cibles pharmaceutiques potentielles. La caractérisation détaillée effectuée sur les différentes enzymes permettra à la conception de tests pour les criblages à haut débit. L'optimisation des conditions de l'activité enzymatique va faciliter l'identification et la caractérisation des inhibiteurs. Prises ensemble, ces études d'approfondissent notre compréhension de la glycolyse/néoglucogenèse dans un groupe diversifié d'organismes.

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Résumé

La majorité des maladies négligées chez l'Homme sont causées par des parasites. Les nématodes parasites ou filaires sont responsables d'un grand nombre de ces maladies, dont certaines sont transmissibles par des arthropodes piqueurs-suceurs comme les moustiques ou les mouches noires. Il est estimé que 150 millions de personnes dans le monde sont infectées par une filaire et plus d'un milliard de personnes sont à risque. Le contrôle des filarioses s'appuie actuellement sur un petit nombre de médicaments qui sont insuffisants en raison de leur faible efficacité et du développement de résistance. Il y a donc un besoin urgent de médicaments nouveaux et améliorés. Depuis quelques années, les symbiotes intracellulaires α-protéobacterien du genre *Wolbachia*, qui sont présents dans la plupart des filaires, ont été étudiés comme une nouvelle approche de chimiothérapie. En effet, ces bactéries sont essentielles pour le développement du ver, sa fécondité et sa survie.

La découverte de médicaments basée sur des cibles précises représente une approche pour la découverte de nouveaux produits thérapeutiques. Nous avons identifié l'enzyme phosphoglycérate mutase co-facteur indépendante (PGMi) comme une cible thérapeutique potentielle chez les filaires et *Wolbachia*. PGMi a précédemment été reconnue comme une cible thérapeutique dans plusieurs parasites protozoaires dont *Trypanosoma brucei*, l'agent causal de la trypanosomiasis Africaine. PGM catalyse l'interconversion du 2 et 3-phosphoglycérate (2- et 3-PG) dans les voies glycolytique et néoglucogénique. Bien que ces voies soient très conservées entre les différents organismes, deux PGMs ont été identifiées, la PGMi et la phosphoglycérate mutase cofacteur dépendante, PGMd. Les mammifères possèdent exclusivement PGMd. Nous avons également identifié l'enzyme glycolytique /néoglucogénique pyruvate phosphate dikinase (PPDK), comme une cible potentielle contre *Wolbachia*. PPDK catalyse la conversion réversible de l'AMP, du phosphoénolpyruvate et du PPi en ATP, Pi et pyruvate. Dans la plupart des organismes, y compris les mammifères, cette activité est assurée par la pyruvate kinase et aucune PPDK n'est présente.

Mon travail de doctorat s'est concentré sur la caractérisation moléculaire et biochimique des PGMis d'un certain nombre d'organismes et de la PPDK de *Wolbachia*, en vue de développer davantage ces enzymes en tant que cibles pharmaceutiques. Des procédures ont été élaborées afin d'optimiser l'expression et la purification de protéines recombinantes. Les PGMis de nématode ont montré des caractéristiques biochimiques similaires, ce qui indique qu'un seul inhibiteur de l'enzyme pourrait être efficace contre toutes les enzymes de nématodes. Les PGMis de *Wolbachia*, *T. brucei* et *E. coli* (et PGMd) ont également été étudiées. Le but ultime de nos études sur la PGMi et la PPDK, est la découverte d'inhibiteurs spécifiques qui pourraient représenter des composés meneurs (lead) pour la poursuite du développement de composés anti-parasitaires. Plusieurs bibliothèques de phages ont été criblées et des peptides qui se lient à la PGMi ont été identifiés. Malheureusement, aucune d'eux n'a montré d'activité inhibitrice.

Abstract

The majority of neglected diseases in humans are caused by parasites. Parasitic nematodes are responsible for a substantial number of these diseases, some of which are transmitted by blood feeding arthropods like mosquitoes or black flies. It is estimated that 150 million people in the world are infected with a filarial nematodes and more than a billion people are at risk. Control of filariasis currently relies on a small number of drugs which are inadequate because of their limited efficacy and development of drug resistance. Therefore there is an urgent need for new and improved drugs. In recent years, obligate α-proteobacterial endosymbionts of the genus *Wolbachia* that are present in most filarial nematodes have been investigated as a new approach to chemotherapy since these bacteria are essential for worm development, fertility and survival.

Target-based drug discovery represents one approach to discovering new therapeutics. We have identified the enzyme co-factor independent phosphoglycerate mutase (iPGM) as a potential drug target in nematode parasites and *Wolbachia*. iPGM has previously been recognized as a drug target in several parasitic protozoa including *Trypanosoma brucei,* the causative agent of African trypanosomiasis. PGMs catalyze the interconversion of 2- and 3-phosphoglycerate (2-PG and 3-PG) in the glycolytic and gluconeogenic pathways. Although these pathways are highly conserved among different organisms, two distinct PGM enzymes are known to exist, iPGM and the cofactor dependent phosphoglycerate mutase, dPGM. Mammals possess exclusively dPGM. We also identified the glycolytic/gluconeogenic enzyme pyruvate phosphate dikinase (PPDK) as a potential *Wolbachia* drug target. PPDK catalyses the reversible conversion of AMP, PPi and phosphoenolpyruvate into ATP, Pi and pyruvate. In most organisms, including mammals, this activity is performed by pyruvate kinase and no PPDK is present.

My Ph.D studies focused on the molecular and biochemical characterization of iPGM from a number of organisms and PPDK from *Wolbachia,* with a view to developing these enzymes further as drug targets. Procedures were developed to optimize expression and purification of recombinant proteins. The nematode iPGM enzymes were found to possess similar biochemical characteristics, indicating that a single enzyme inhibitor would likely be effective against all nematode enzymes. iPGM enzymes from *Wolbachia*, *T. brucei* and *E. coli* (and dPGM) were also studied. The ultimate goal of our studies on iPGM and PPDK is the discovery of specific inhibitors that may represent lead compounds for further development as anti-parasitic compounds. Several phage display libraries were screened and peptides which bind to iPGM were identified. Unfortunately, none displayed enzyme inhibitory activity.