“Structure function aspects of a PAS domain containing phosphoglycerate kinase from *Leishmania major*”

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By
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"Dedicated to my parents, to my teachers and to all my well-wishers....."

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Saroj Biswas

Dated

PREFACE

The first evidence of the presence of genus *Leishmania* was found in the fossil amber from Mesozoic era. In 15th century, Pedro Pizarro outlined the demolition of the nose and lips of the agriculture workers working on the lower eastern slopes of the Andes. Alexander Russell made a key progress in the discovery of Leishmaniasis after examining a Turkish patient and named it as, "Aleppo boil" in 17^{th} century. Later, the disease is known as leishmaniasis. William Leishman and Captain Charles Donovan named one of the earliest strains of *Leishmania* (*Leishmania donovani*) which causes the visceral leishmaniasis, was popular with different names like kala azar, dum-dum fever, espundia and white leprosy.

Leishmaniasis is a highly disabling disease found in the tropics, subtropics and southern Europe. It is caused by as many as 30 different species of the genus *Leishmania*. About 12 million people are affected every year of which 50,000 to 60,000 deaths occur annually. Common medications for leishmaniasis include paromomycin, flucanazoles, combination of pentavalent antimonials, pentamidine, miltefosine, amphotericin B etc.All these drugs are very expensive and they have several side effects. Besides, no vaccine has been discovered as of now. Another very important issue is the co-infection of *Leishmania* with *Mycobacterium* or HIV. Such co-infections can be fatal. Hence, it is a need of the hour to discover new drugs and in order to do so it is an absolute requirement to identify new potential drug targets.

The survivability of an organism depends on how it responds to the alterations in the internal and external environment. All organisms have evolved with some sophisticated tools to identify these alterations. One such is the evolution of sensory proteins. These types of proteins conventionally carry a sensory domain which allows them to sense particular changes in the surrounding environment. Last 5 decades, researchers have identified diverse sensory domains which are capable to recognise a wide variety of internal and external cues like pH changes, voltage changes, nutrient availability, oxygen, redox potential, light etc. In the early 1990's some scientists recognised a versatile sensory domain while studying Drosophila proteins. The domain was named as PAS domain, after the three proteins (Per-Arnt-Sim) were discovered. Later on, researchers found that PAS domain containing proteins are present in all form of organisms. These proteins are acts as signalling domains in broad range of enzymatic and non-enzymatic proteins. PAS domain shows less sequence homology but it shows high structural homology and acts differently in different organisms. In prokaryotic organisms, PAS domain helps in heme binding and oxygen sensing, pH sensing, divalent cation sensing, nutrient sensing in metabolism, redox potential sensing, and visible light sensing. While in eukaryotes it helps in hypoxia response pathway, dioxin response pathway.Though PAS domain containing proteins are frequent in archaea, bacteria and eukarya yet they

are still not reported in the trypanosomatid parasites *Trypanosoma* and *Leishmania*. Our group first time identified and characterized a unique PAS domain containing phosphoglycerate kinase (LmPAS-PGK) in the unicellular eukaryotic organism *Leishmania major*. This protein is absent in the host and it plays an important role in cell survivability under nutrient stress and Mg^{2+} stress condition which make it a potential target for drug discovery. The protein is activated by acidic pH 5.5. Most of the PGK show their optimum activity at neutral pH 7.5 whereas PAS domain containing LmPAS-PGK exhibit its optimum activity at acidic pH 5.5. PAS domain plays a crucial role in switching the PGK catalytic activity. It makes us curious to know the molecular mechanism, how PAS domain interacts with catalytic PGK domain through cofactor Mg2+ binding.

The major work in this dissertation is to explore the structure-function aspect of PAS domain containing phosphoglycerate kinase through generating various mutants of the enzyme. An effort is made to explore the mechanism of regulation by PAS domain at the molecular level. The findings of our work are discussed in three consecutive chapters. Chapter 1 discusses about higher concentration substrate (ATP) as well as product (ADP) inhibits wild type LmPAS-PGK activity. Chapter 2 deals with role of cofactor Mg^{2+} ion and other divalent cations in LmPAS-PGK. Chapter 3 tells about role of histidine 57 in the repression of PAS domain mediated catalytic PGK domain.

ABBREVIATIONS

ADK	Adenosine kinase
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
APS	Ammonium persulfate
APX	Ascorbate peroxidase
ATG	Autophagy related genes
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CM	Complementary cells
CPB	Cysteine peptidase B
CT	Control cells
EDTA	Ethyl diamine tetra acetic acid
FACS	Fluorescence activated cell sorter
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3 phosphate dehydrogenase
LmPAS-PGK	PAS domain containing phosphoglycerate kinase from *Leishmania major*
HKO	Half knockout cell
Hr	Hour
IPTG	Isopropyl β-D-thiogalactopyranoside
Kb	Kilo base
KO	Knockout cells
kDa	Kilo Dalton
MES	2-(N-morpholino) ethanesulfonic acid
Min	Minute
mg	milli gram
ml	milliliter
mM	milli molar
NAD^+	nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
nm	nano meter
nM	nano molar
OD	Optical density
OE	Overexpressing cell
PAS	Period, Arylhydrocarbon nuclear transporter, Single minded
PCR	Polymerase Chain Reaction

PDE	Phosphodiesterase
PGK	Phosphoglycerate Kinase
PKC	Protein kinase C
PI	Propidium iodide
PMSF	Phenyl methyl sulphonyl fluoride
RNaseA	Ribonuclease A
RPM	Revolution per minute
TAE	Tris acetate EDTA
TEA-HCl	Triethanolamine hydrochloride
TEMED	Tetramethylethylenediamine
Tris	Tris (hydroxymethyl) aminomethane
UV	Ultra violet
VP1	Vacuolar pyrophosphatase 1
µl	Microlitre
µm	Micrometre
1,3- BPG	1,3 bisphosphoglycerate
2-DG	2- deoxy D- glucose
3 PGA	3- Phosphoglyceric acid
ADK	Adenosine kinase
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
APS	Ammonium persulfate
APX	Ascorbate peroxidase
ATG	Autophagy related genes
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Kb	Kilo base
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kDa	Kilo Dalton
MES	2-(N-morpholino) ethanesulfonic acid
Min	Minute
mg	milli gram
ml	milliliter
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NAD^+	nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
nm	nano meter
nM	nano molar
OD	Optical density
OE	Overexpressing cell
PAS	Period, Arylhydrocarbon nuclear transporter, Single minded
PCR	Polymerase Chain Reaction
PDE	Phosphodiesterase
PGK	Phosphoglycerate Kinase
PKC	Protein kinase C
PI	Propidium iodide
PMSF	Phenyl methyl sulphonyl fluoride
RNaseA	Ribonuclease A
RPM	Revolution per minute
TAE	Tris acetate EDTA
TEA-HCl	Triethanolamine hydrochloride
TEMED	Tetramethylethylenediamine
Tris	Tris (hydroxymethyl) aminomethane
UV	Ultra violet
VP1	Vacuolar pyrophosphatase 1
µl	Microlitre
µm	Micrometre
1,3- BPG	1,3 bisphosphoglycerate
2-DG	2- deoxy D- glucose
3 PGA	3- Phosphoglyceric acid

CONTENTS

Introduction

INTRODUCTION

1.1. Outline of *Leishmania* and leishmaniasis:

Leishmaniasis is a highly disabling disease found in the tropics, subtropics and southern Europe [1]. It is caused by the intracellular protozoan parasite of the genus *Leishmania*. The parasite is transmitted to humans by the bite of mainly two types of sand fly, *Phlebotomus* and *Lutzomyia* [2]. Leishmaniasis is considered as one of the "most neglected diseases," based on the limited resources invested in diagnosis, treatment and control, and its strong association with poverty. According to the World Health Organization [3], leishmaniasis is an important tropical disease if it is not properly managed can have potentially detrimental outcome. The symptoms of leishmaniasis usually depend on the infecting *Leishmania species* and the nature of the host immune response. About 12 million people are affected worldwide by 20 different species of *Leishmania* [1].

1.1.1 Taxonomy of *Leishmania*:

Systematic classification of *Leishmania* according to [4]

Kingdom: Protista
- Subkingdom: Protozoa
- Phylum: Sarcomastigophora
- Class: Zoomastigophora
- Order: Kinetoplastida
- Sub order: Trypanosomatina
- Family: Trypanosomatidae
- Genus: *Leishmania*

Historically*Leishmania* classification was based on the ecobiological criteria such as vectors, geographical distribution, tropisms, antigenic properties and clinical manifestations. Now varieties of molecular biological techniques have been introduced for the selection of complex species, sub species and strains and these techniques are proved to be more reproducible, authentic and reliable. World Health Organization published a taxonomic scheme for the use of these molecular techniques. To date, among 30 known species, approximately 20 are proved to be pathogenic for humans. These species are recognized by their different epidemiological and clinical characteristics, related to different genetic and phenotypic profiles. The molecular techniques for classification of different types *Leishmania* at genus, species and strain level, are given in Table 1.

Table.1. Molecular tools for classifying *Leishmania.*

Level of differentiation	Technique used
Genus	Nuclear DNA restriction fragment patterns Amplification of variable sequences of the ssu rRNA Comparison of DNA and RNA polymerase gene sequences
Species	Multilocus enzyme electrophoresis (MLEE) PCR based amplification of kinetoplast mini-circle DNA (KDNA)
Strain	PCR fingerprinting and RAPD (random amplified polymorphic DNA) techniques Multilocus microsatellite typing (MLMT) Multilocus sequence typing (MLST)

1.1.2. Epidemiology of leishmaniasis:

The oldest DNA of *Leishmania donovani was* found inthe specimen taken from ancient Nubian and Egyptian mummies. Primeval artifacts retrieved from Africa, America and middle east prove the happening of old world cutaneous leishmaniasis which was also known as oriental sore [4]. Currently 12 million cases of leishmaniasis exist globally as reported by World Health Organisation. 2 million new cases arise every year where 0.5 million people affected with fatal visceral leishmaniasis and the rest with cutenous leishmaniasis [5].

Leishmaniasis is found in people in tropical areas of approximately 90 countries in the tropics, subtropics and southern Europe (Fig.1 and Fig.2). The ecologic settings range from rain forests to deserts. Usually, Leishmaniasis is more common in rural areas than in urban areas. Deforestation, climate change and other environmental changes have the potential to expand the geographic range of the sand fly vectors and areas in the world where leishmaniasis is found. Leishmaniasis is found in people on every continent except Australia and Antarctica.

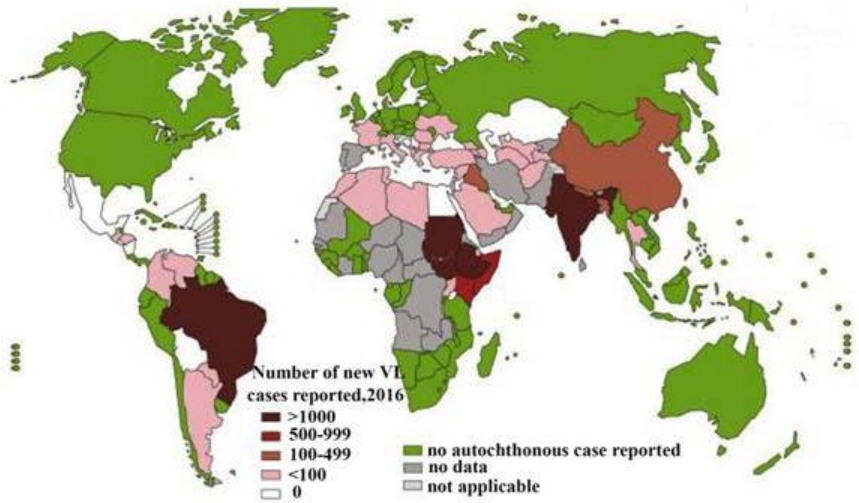

Fig.1. Endemicity of cutaneous leishmaniasis globally (Adapted from www.who.int)

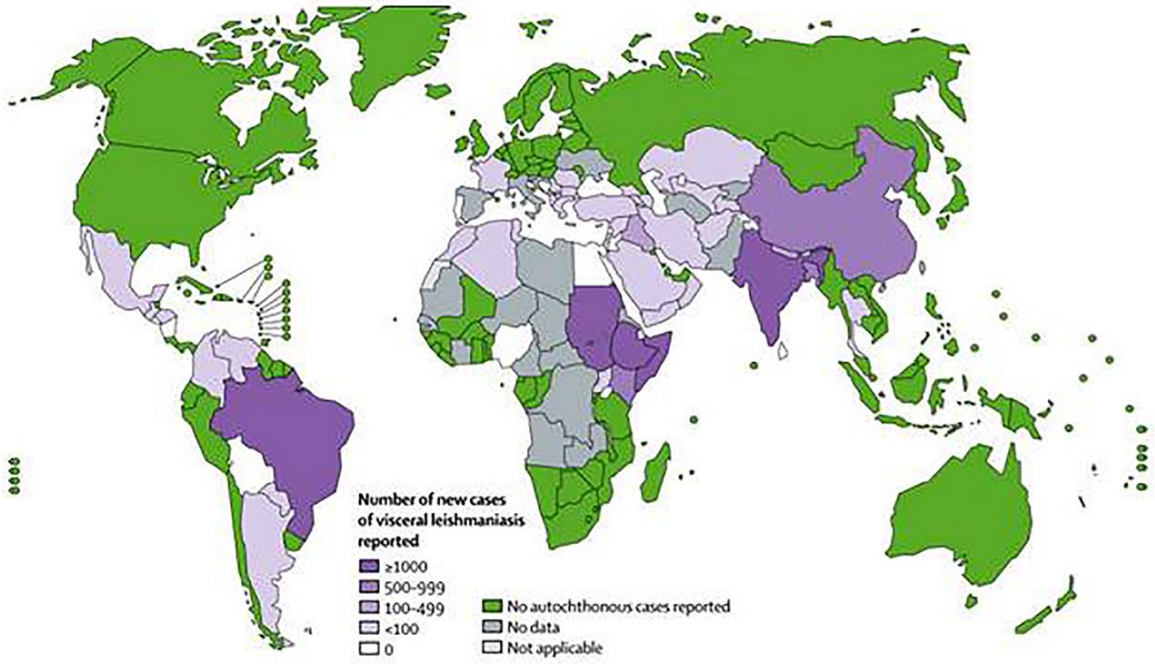

Fig.2. Endemicity of Visceral Leishmaniasis globally (Adapted from www.who.int)

1.1.3. Different form of leishmaniasis:

Depending on the various clinical symptoms, leishmaniasis can be divided into four types. 1) Visceral leishmaniasis (VL) or kala-azar; 2) cutaneous leishmaniasis (CL); 3) muco cutaneous leishmaniasis; 4) Post Kala Azar Dermal Leishmaniasis (PKDL).

1.1.3.1. Visceral leishmaniasis (VL)

The common name of visceral leishmaniasis is Kalazar or black fever (www.who.int). Visceral leishmaniasis is known as most severe form of leishmaniasis, without proper diagnosis and treatment it can be highly fatal [6]. The parasite relocates to the internal organs such as the liver, spleen hence it is called visceral leishmaniasis. It is mainly prevalent over large parts of south and east Asia (mainly in India, Bangladesh and Nepal), a large part of Africa, the southern Europe, and South America.Two species of *Leishmania*are known to give rise to the visceral form of the disease. The species commonly found in East Africa and the Indian subcontinent are *L. donovani* and the species found in Europe, North Africa, and Latin America are *L. infantum*, also known as *L. Chagasi* [7].The most common symptoms of visceral leishmaniasis (Fig.3) are fever, blackening of the skin, splenomegaly, hepatomegaly and the other symptoms are very easy to mistake with malaria. This type of leishmaniasis is highly fatal if co-infected with HIV.

Fig.3. Visceral leishmaniasis

1.1.3.2. Cutaneous leishmaniasis (CL)

The common form of leishmaniasis which affects humans is cutaneous leishmaniasis [1]. This form of leishmaniasis is disseminated through the sand fly (phlebotomine) bite which is responsible for skin infection. Around twenty various species are known that are the causative agent of cutaneous leishmaniasis. Promastigotes are circulated to humans by the sandfly bite, which then invades macrophage and replicates intracellularly. Skin developed with uplifted red lesions (Fig.4) at the fly biting site (weeks to years). The lesions are prone to secondary infection through different bacteria. In many species, it is shown that the wound may spontaneously heal with scarring, but later shows up in a different place. Cutaneous leishmaniasis is widespread around 70 nations across the globe, largely in Brazil, Peru, Afghanistan, Pakistan, Saudi Arabia, Algeria, and Syria. The main causative species are *L. major, L. infantum, L. amazonensis, L. peruviana, L. panamemsis, L. aethiopica* [8].

Fig.4. Cutaneous leishmaniasis

1.1.3.3. Mucocutaneous leishmaniasis

Mucocutaneous leishmaniasis generally occurs after the evident curing of cutaneous infection [9]. It produces destructive and disfiguring lesions of the face. Lesions can appear with in 2 to 30 years after the cutaneous infection [10]. The infection spreads either through blood or the lymphatic system [11]. Mucocutaneous leishmaniasis is most often caused by *Leishmania braziliensis*, but some cases caused by *L. aethiopica, L. panamensis, L. guyanensis* have also been reported [12]. Although, the nasal and oral cavities are primarily affected but lesions may extend upto the oropharynx and thetrachea (Fig.5) [11]. 90% of mucocutaneous form of leishmaniasis takes place in South American regions.

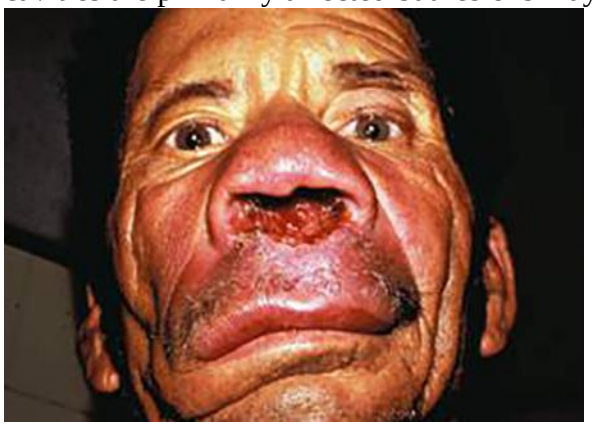

Fig.5. Mucocutaneous leishmaniasis

1.1.3.4. Post Kala Azar Dermal Leishmaniasis (PKDL)

The complicated form of Visceral leishmaniasis is well known as post kala-azar dermal leishmaniasis (PKDL) which is the reappearance of kala-azar as a macular, nodular and papular form on the

skin of previously affected persons around twenty years after treatment [13, 14]. PKDL is majorly found in the arm, face and different upper parts of the torso. It is mostly found in Sudan and India, in Sudan PKDL interval follows up to 6 months whereas in India it follows nearly 2 to 3 years. 60% of treated cases found to be PKDL in Sudan, in case of the Indian variant of PKDL, it shows enlargements of nodules, form plaques, ulceration occurs rarely. But, in the African variety ulceration is frequent as the disease progresses (Fig.6). The nervous system is also affected by the African variety but, it is rarely found in the Indian subcontinent [15]. The histology analysis of PKDL illustrates combinations of chronic inflammatory cells, macrophage and epithelial granuloma [16]. Earlier diagnostic tools used in PKDL are less sensitive. The new diagnostic approaches include a) illustration of the in-vitro culture under the microscope, b) Immuno-diagnosis, c) identification of parasite DNA from tissue. Current PCR based sophisticated tools have much more sensitivity and specificity. The appearance of PKDL has been outlined in several HIV affected patients which may be a problem in upcoming future.[17].

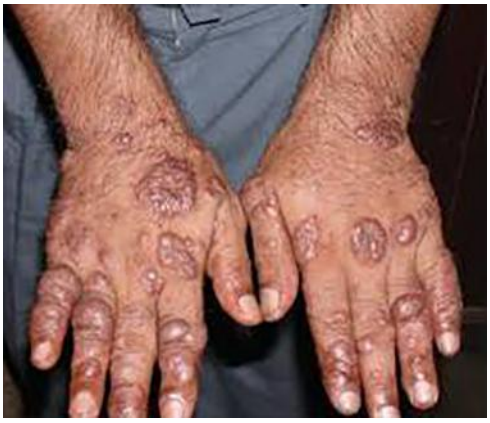

Fig.6. Post Kala Azar Dermal Leishmaniasis

1.2. Life cycle of *Leishmania*

Leishmania are intracellular protozoan pathogen, digenetic parasites which alters between promastigote and amastigote form in an invertebrate host (mid gut of sand fly vector) and a vertebrate host (macrophage), respectively. Along with human, rodents, edentates, canids are also known for reservoir host of *Leishmania* [18]. In the promastigote form the parasite is a bit elongated and motile with sole anterior flagellum. Fully developed body length in

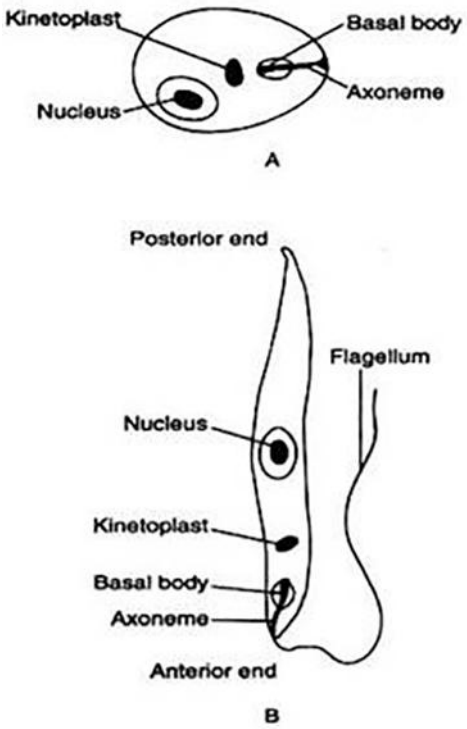

Fig.7. Diagramatic representation of *Leishmania* amastigote and promastigote along with nucleus and kinetoplast (adapted from researchgate)

this form are 15- 30 µm, 1-2 µm in width. In this form a single nucleus and a kinetoplast is present transversely close to the anterior end (**Fig.7**). *Leishmania* is phagocytised by macrophage and develop into non-motile intracellular amastigote in vertebrate host. Amastigotes are moderately round to oval in shape and 3-7 µm in diameter. It contains a single nucleus and kinetoplast, but its remain only a elementary flagellum [19]. In both forms they are able to replicate via binary fission. *Leishmania* life cycle starts while an infected female sand fly injected infective form of promastigote into a healthy individual during blood meal. These promastigotes are instantly phagocytosed by macrophages which act as a cellular fence to infection. Being a digenetic intracellular pathogen, *Leishmania* are able to survive in the host phagolysosome and are capable to differentiate into amastigote via binary fission and multiplies until the lyses of macrophage and amastigotes are released to the flow circulation. Through the circulation these amastigotes conquer blood monocytes, macrophages of the spleen, liver, lymph nodes, bone marrow and tissues of the reticuloendothelial (RE) cells. Once the parasite has entered the systemic circulation, it can once again be taken up during a blood meal by the female sand fly (Fig.8). The amastigotes are transformed into flagellated promastigote in the sand fly midgut by a succession of flagellated intermediate, which takes up to 3 days [20]. These flagellated form of parasites subsequently migrate to pharynx and buccal cavity of the sand fly after 6 to 9 days [1]. Then the bite of these sand flies spread the infection to susceptible persons and the life cycle of *Leishmania* is repeated [21].

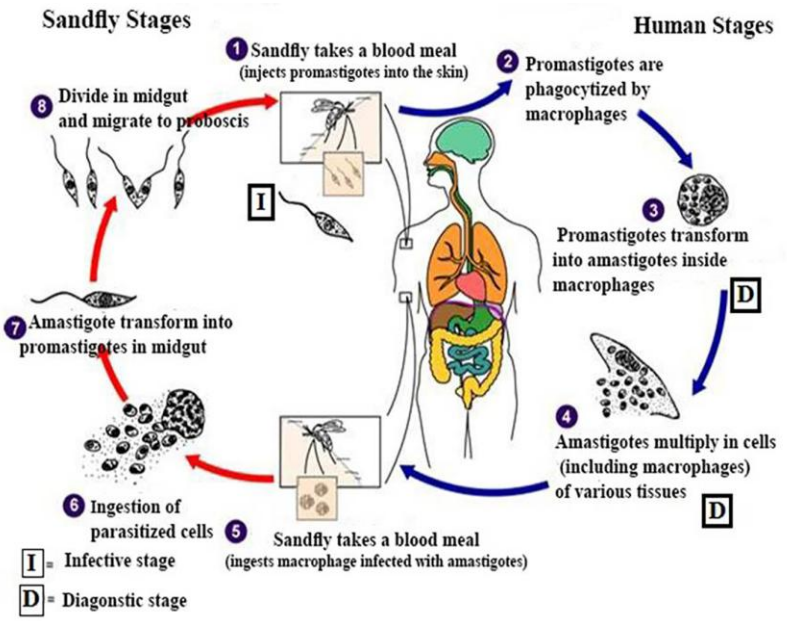

Fig.8. Life cycle of Leishmania (Adapted from www.cdc.gov)

1.3. Treatment and prevention against Leishmaniasis

The best approach to secure someone from infection is to protect themselves from sand fly bites. There are some drugs which used to treat against leishmaniasis. But these available medications are expensive, toxic and ineffective due to drug resistance. Therefore it is very important to develop a new drug target to protect the disease. Various drugs and vaccines have been under assessment in clinical trials which exhibits auspicious therapeutic claim.

Till now among various compounds and different formulations have showing antileishmanial activity. Out of them only a few have been proven worthy in clinical uses (Table 2). The first line drugs used for the treatment against leishmaniasis are pentavalent antimonials including sodium stibogluconate and meglumine antimoniate although the uses of these drugs are limited toxicity and drug resistance. Second line drugs against leishmaniasis such as pentamidine, amphotericin B, miltefosine and paramomycin are effective but they have different after effects [22, 23].

Table:2. Current scenario of available antileishmanial drugs. Adapted from: Bhargava P. et al., Ref. [24]

Drug	Properties and administration	Comments	Adverse effects
Pentavalent antimonials	Polymeric organometallic complexes, intravenous, or intramuscular For VL and CL	Drug resistance in Bihar, India. Variable response indifferent forms of CL. Generic sodium stibogluconate (SSG) has made treatment cheaper Pain, erythema,oedema, abdominal pain, nausea, and thrombocytopenia Amphotericin B Polyene antibiotic,fermentation product of*Streptomyces nodus*,intravenous.	Pain, erythema, oedema, abdominal pain, nausea, and thrombocytopenia
Amphotericin B	Polyene antibiotic, fermentation product of *Streptomyces nodus*, Intravenous	For VL, CL, and complex forms of CL, for example, MCL.first-line drug for VL in India where there isantimonial resistance.	Infusion related, azotemia, anemia, or hypokalemia
Miltefosine	Hexadecylphosphocholine, oral	First oral drug for VL.Effective against some forms of CL contraindicated inpregnancy.	Nausea, vomiting and/or diarrhea, raised creatinine, and raised LFT's
Paromomycin	Aminoglycoside (also known as aminosidine ormonomycin), fermentation product of *Streptomyces rimosus*. Supplied as sulphate. Intramuscular for VL and topical for CL	Registered for VL in India, completed Phase III trials forVL in East Africa where less effective in Sudan. Topical formulation (12%) with methyl benzethonium chloride available for CL. Topical with gentamicin and surfactants in Phase III trial.	Pain, erythema, oedema, blisters, and ototoxicity
Pentamidine	Diamidine, as isethionate salt, intramuscular	For specific forms of CL in South America only.	Nausea, vomiting, diarrhea, hyperglycemia, and cardiotoxicity.

To connect with the highly demand of new antileishmanial medication, the focus has turned on to several new class of chemotherapeutic agents like antileishmanial synthetic compounds, antileishmanial natural products which may be isolated from forest or marine sources and immunomodulators. Several other antileishmanial compounds are under clinical trial like 8-aminoquinolone, stimaquine which is alternative oral drug in phase II trial, ketoconazole, fluconazole, itraconazole (antimycotic azoles) [25]. Advancement in modern bioinformatics provides a new genome based approach for new drug discovery. This advance genomic methodology incorporated with combinatorial chemistry and high throughput screening [26] leads to a large scale drug discovery effort touching on development of new antileishmanial drugs [23, 27].

The most economical approach for the prevention of infectious diseases is vaccination strategy. Thus, development of vaccines against leishmaniasis is the ultimate and long term goal in veterinary and human medicine. Till now there is no vaccine is present against leishmaniasis across the world. Development of effective vaccines against *Leishmania* is hampered due to antigenic diversity and digenetic life cycle of *Leishmania*. In the past two to three decades a lot of research has been conducted on the production of vaccines against *Leishmania* and several vaccine preparation are in different stages of clinical trials (**Table 3**) [28, 29].

Table 3: Vaccines for Leishmaniasis

Generation	Nature	Present status
First generation	Killed parasites that trigger low immune response.	Phase III clinical trials in South America and Asia since 1940 [28]
Second generation	Genetically, modified live parasites, bacteria or viruses having recombinant or native fractions of*Leishmania* genes.	Antigens like recombinant hydrophilic acylated surface protein B1, *Leishmania* homolog of receptors for activated C-kinase [30], kinetoplastid membrane protein-11, A2 protein, Histone H1 [31] from *Leishmania* have been used in vaccine development with considerable success.
Third generation	Composed of DNA vaccines which are more stable, cheaper and induce stronger and more durable immune responses through triggering innate immune responses.	Still under development and standardization.

1.3.1. Drug resistance in Leishmaniasis

The first successful drug against leishmaniasis was discovered by professor U.N brahmachari in 1912 (urea stibamine). Refinement of drug therapy guidelines for new and old world leishmaniasis crucially is needed on a universal basis because treatment failure is a growing problem. The rudimentary reason for treatment failure is drug

resistance. Global HIV/AIDS epidemic also contribute to this phenomenon along with its impact on the immune system [32]. Drug resistance in *Leishmania* depends on two factor a) Host factor, b)*Leishmania* related factor.

a) Host factor- i> host immune status, ii> genetic make up, iii> pharmacokinetic properties of drug.

b) *Leishmania* related factor- i> physiological events leads to drug resistance [33], ii> Elevated levels of intracellular proteins such as ornithine decarboxylase, glutamylcysteine synthetase (GCS), P-glycoprotein (PgpA) and decreased level of sb reductase and even chronic exposure to arsenic in drinking water [34] are thought to be involve in antimony resistance in *Leishmania*. Extensive use of second line medications like miltefosine, amphotericin B to tackle antimony resistance leishmania also leads to resistance to these drugs. Amphotericin B resistant *Leishmania donovani* promastigotes exhibited, reduced influx and increased efflux of the drug which also make significant alteration of plasma membrane sterol profile by replacing ergosterol with cholesta-5,7,24-trien-3β-oldue to loss of function of S-adenosyl-L-methionine-C24-Δ-sterol-methyltransferase (SCMT) [35]. Progress of miltefosine resistant *L.donovani* is outlined to be the result of inactivating mutations on a novel plasma membrane P-type transporter (LDMT) gene [36]. However, in pentamidine resistant *L. amazonesis* alternations in membrane fluidity, lipid content and loss of pentamidine binding sites was demonstrated [37] (Fig.9).Excluding the finding of a new and effective antileishmanial drugs, special attention needs to be taken to control the development and spread of drug resistance by monitoring the drug response and occuerence of resistance.

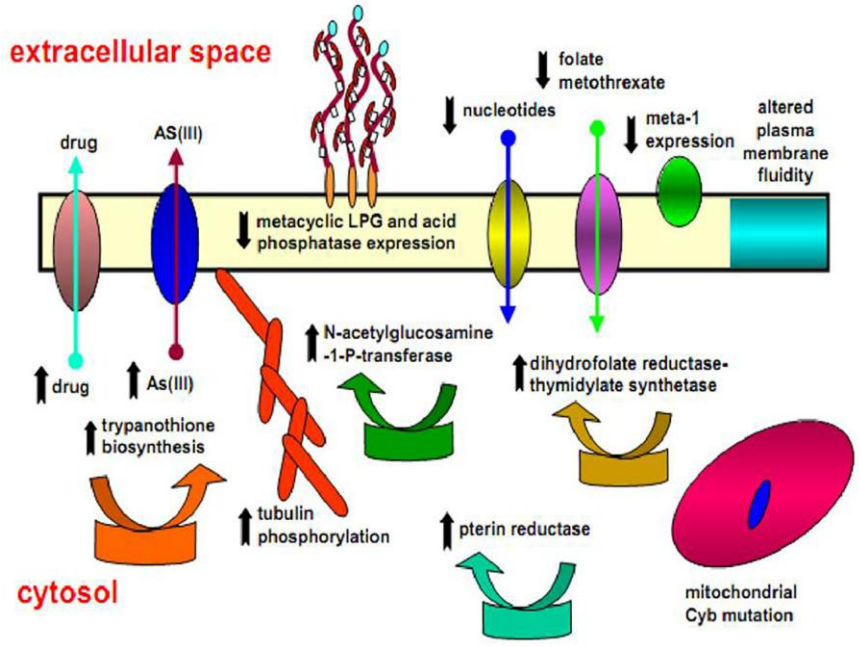

Fig.9.The physiological events associated with drug-resistance in *Leishmania*. Black arrows indicate whether a given parameter is increased or decreased in the resistant parasite. (Adapted from: A Ponte-Sucre Kinetoplastid Biology and Disease, 2003 Springer)

1.3.4. Role of phosphoglycerate kinase in *Leishmaia*

Phosphoglycerate kinase (PGK) is a well-studied and pivotal enzyme in *Leishmania*. It is a crucial glycolytic enzyme which is highly conserved. PGK is responsible for conversion of 1, 3 bisphosphoglycerate and ADP to 3 phosphoglycerate and ATP (Fig.10). It involves in the first ATP producing step in glycolysis. Apart from glycolysis, PGK is reported to be a moonlighting protein which engaged in regulating autophagy [38, 39], helping DNA repair [40], prevention of angiogenesis during cancer [41] and controlling spermatogenesis [42]. Previously reported that *Leishmania* contains two phosphoglycerate kinases. One located in the cytosol which is called PGKB and the other oneis located in glycosome which is called PGKC. While, both the PGK's are expressed in the promastigote and amastigote forms, still the cytosolic PGK accounts for 80% of the total PGK activity [43, 44]. Peroxisomal targeting sequence (PTS) is present at C-terminus end of PGKC. This signalling sequence helps to impact this isoenzyme to the glycosomes. Genome bioinformatics analysis stipulates the presence of an unique uncharacterised phosphoglycerate kinase. This unique enzyme is present in all the *Leishmania* species and in few *Trypanosoma.* This enzyme has unusual (1-120) amino acids in the N-terminus which shows low pairwise sequence homology to PAS (Per-Arnt-Sim) domain, while other domain has about 70% sequence homology to PGK.

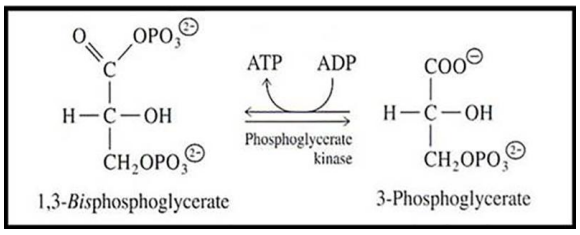

Fig.10. Reaction catalysed by phosphoglycerate kinase (Adapted from: web.campbell.edu)

1.5. Overview of PAS domain

PAS (Per- Arnt- Sim) domains were first identified by sequence homology in the Drosophila proteins. Per means "Period circadian protein", Arnt denotes "Aryl hydrocarbon receptor nuclear translocator protein", Sim represents "Single minded protein". PAS domains are sensory domain which are widely present in proteins from all kingdom of life forms [45]. The PAS domain are generally 100-120 amino acids long and are connected to a variety of enzymatic and non-enzymatic effector modules which take parts in different important cellular signalling cascade[45]. PAS domain shows very low sequence homology, although the solved crystal structures of around 12 PAS domain containing protein reveals that they share notably conserved three dimensional structure [45]. PAS domain contain 4-5 antiparallel beta sheets flanked by several alpha helix [46]. Apart from other sensor modules, PAS domain containing proteins are generally located in cytosol. The PAS domain in various PAS containing proteins accomplish a diverse functions such as protein-protein interaction [47-49], signal transduction [50], sensing internal and external stimuli [51]. Due to sequence variation PAS domain can able to bind with extensive variety of ligands and co-factors. Depending on its structure, ions and small molecules that bind to the PAS domains can provide a direct signal [52-54] or

previously bound co-factors are also able to sense redox potential [55-58], visible lights [59, 60], dissolved gases [61, 62]or voltage[63].

1.5.1. Nomenclature of the PAS domain

By differentiating the sequence of Drosophila clock protein PERIOD, vertebrate aryl hydrocarbon nuclear translocator (ARNT) and the developmental regulator single minded (SIM), hence it is called Per-Arnt-Sim or PAS [64, 65]. Two ~70 amino acid residue repeats named as PAS A and PAS B found in a single protein [66]. After few years PAS repeats were found in the clock genes of cyanobacteria [67] and from the sensor kinases of bacteria [68]. PAS repeat like pattern was also recognised in an algal phytochrome and in 20 different protein from both eukaryotes and prokaryotes [69]. This pattern is indistinguishable to the common fold present in the N-terminus of photoactive yellow protein [70]. The crystallographic structure of the photoactive yellow protein was solved before. Different reviews in the literatures narrate the evolution and understanding of the PAS domain. Briefly it was report as a PAS repeat, PAS/PAC motif or S1/S2 box is now known to form a single coherent protein fold of nearly 100 amino acid residues [51].The fold is organised of five antiparallel beta sheet with 2-1-5-4-3 strand order, alpha helix intervene to the beta sheet and create pockets where ligand binding occurs [45]. In following studies they were found nearly 200 proteins from different organisms throughout the phylogenetic tree [71, 72]. Though the term PAS repeats and PAS domain were initially think different but slowly the distinction was omitted between two [66]. Widely it is considered as PAS domain now.

1.5.2. Structure of PAS domain

The first solved three dimensional structure of PAS domain was the photoactive yellow protein (PYP) from *Halorhodospira halophile* (Fig. 11). The solved structure shows that the globular fold containing around 100 amino acids. Pinpointing of PAS domains are a bit tough as they show very low pairwise sequence identity which is roughly 20% on an average. Though, new PAS domains are generally identified by sequence comparison with known PAS domains or by advanced bioinformatics search tools like PSI-BLAST [73] or HMMER [74]. The canonical PAS fold appears the α/β structure comprising of five antiparallel β strands namely A_β, B_β, G_β, H_β, I_β and flanked by four α helix (C_α, Dα, Eα,Fα) (Fig.11) [46]. Each PAS domain is further distinguished by a very degenerate

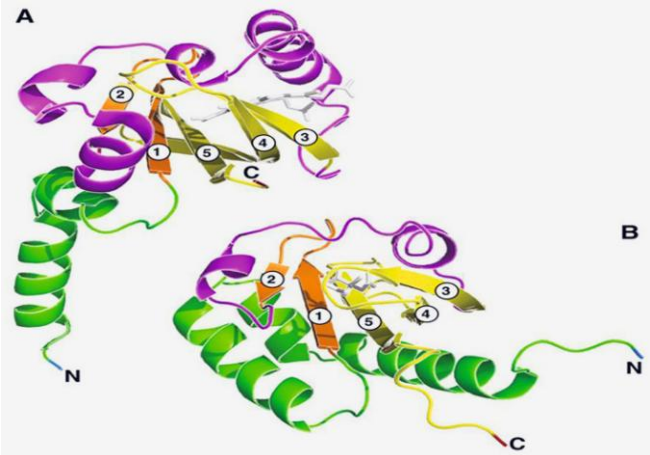

Fig.11. Structure of PAS domain (PDB ID: 1D06-A & PDB ID: 1P0Z-B)

around 50 amino acid repeat with an conserve phenylalanine, histidine and aspartic acid at positions 1, 41 and 44 respectively [75, 76]. Whether one protein holds multiple PAS domain then they are labelled alphabetically as PAS-A, PAS-B, PAS-C and so on from the N- terminus to C-terminus [46].

1.5.3. Function of PAS domain

Pas domain containing proteins are cellular signalling cascade which act as signalling protein. PAS domains are flexible sensors that can bind and also sense tiny endogenous molecules such as cellular metabolites, molecular oxygen, polyaromatic hydrocarbons, divalent cations (Mg^{+2}, Mn^{+2}, Ca^{+2}, Zn^{+2}), heme, flavin nucleotides etc. It is found that PAS domain containing proteins play crucial role in regulating hypoxia responses dioxin response pathways, circadian rhythm. These regulation systems are overall adaptive response of the whole organism [66]. PAS-A domain is responsible for protein/protein interaction in the dioxin response pathway. PAS-A domain is deleted from Ahr, as a consequence it decrease its interaction with Arnt and successively inhibits the initiation of transcription [77-79]. In comparison with PAS A domain, PAS-B domains are more versatile. Apart from participating in protein/protein interaction with different types of proteins PAS-B domains can also sense small molecules [80-84]. PAS domain proteins are able to sense environmental stimuli (Fig.12). The role of PAS domain containing proteins in prokaryotes and eukaryotes are elaborated below.

Fig.12. Common functions of PAS domain containing proteins

1.5.4. Functions of PAS domain in prokaryotes

1.5.4.1. Heme binding and oxygen sensing

α-proteobacteria maintain a sensor histidine kinase, FixL, which modulates the expression of several important genes in microaerobic and anaerobic condition [85-87]. It is able to sense oxygen moderated by a heme cofactor [88]. A related heme binding PAS domain containing direct oxygen sensor, DosP, is found in *Escherichia coli* [89]. The enzyme is an oxygen regulated phosphodiesterase which catalyses the transformation of cyclic –di-GMP into pGpG [62, 90]. The heme ligation in dosP-PAS1 and FixL-PAS is conserved a histidine residue at a proximal site (Fig.13). This conservation shows evidence for evolutionary divergence in molecular level of heme binding in a common ancestory.

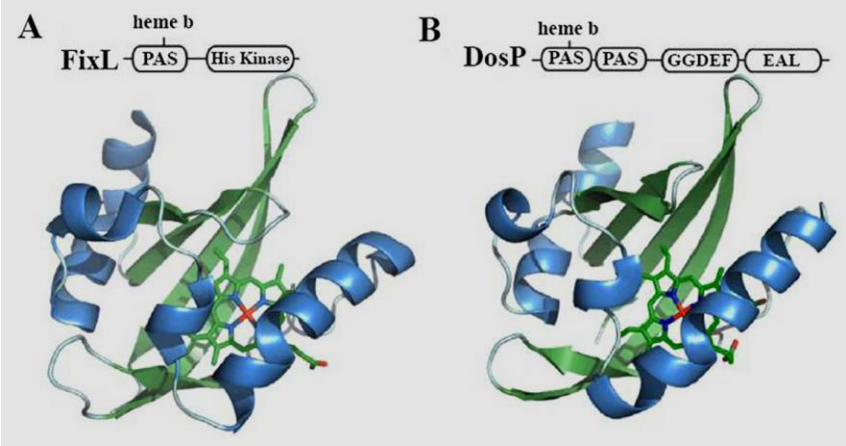

Fig.13. Heme binding PAS domain. A) FixL protein of *B.japonicum* (PDB ID: 1DRM). B) DosP protein of *E.coli* (PDB ID: 1S66) (Adapted from Henry et al, Annu Rev Microbiol., 2011)

1.5.4.2. Role in pH sensing

All organisms sense and responds to extracellular and intracellular pH changes which impacts a vast number of key processes like metabolic reactions, cell motility, ATP synthesis, gene expression, apoptosis. The structure, stability, interactions of proteins are determined by charges present on diverse group which highlighting the perquisite for pH homeostasis. pH sensing has been deliberated in various context which includes blood pH homeostasis [91], sperm activation [92], bacterial chemotaxis receptors [93]. pH sensing by biological macromolecule depends on its side chains. Aspartate, glutamate and histidine plays acrucial role in pH sensing because these amino acids have pKa value near neutral pH. Changes in protonation of important residues lead to conformational alterations which confer changes in signalling states. Histidine kinase of *H. pylori* (HP165(ArsS)) in which His 94 residue has been recognised as crucial for pH sensitivity [94]. *H. pylori* is an acid tolerant gastric pathogen which are able to sense pH changes by a chemoreceptor and its molecular mechanism is given by in Fig.14. *H. pylori* is capable to recognise and give its feedback to chemicals in its environment, where pH is an crucial nod that regulates its distribution [95]. Periplasmic section of TlpB of *H. pylori* reveals a homodimer that contains PAS domain where urea molecule binds with the PAS domain in canonical ligand binding site. This binding is sensitive to pH changes. The key residue is responsible in pH sensing by an aspartate (Asp114) where urea binding occurs through specific affinity depended manner.

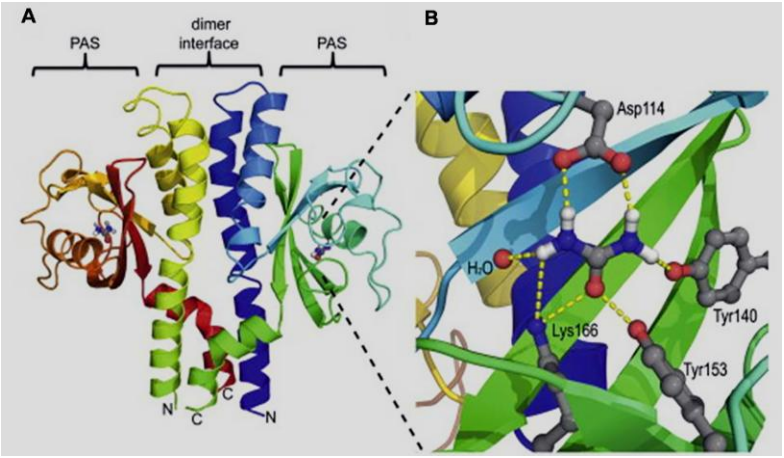

Fig.14. Role of PAS domain in pH sensing. (PDB ID: 3UB6) (Adapted from Structural Basis of *H. pylori* Acid Chemoreception)

Biophysical studies and mutational analyses of the specific binding of urea bear a mechanism where the cofactor urea binding in a pH sensitive fashion (Fig.14)[96].

1.5.4.3. c) Role in divalent cation sensing

In different organisms, it is found that PAS domain also able to bind with different divalent cations and regulates protein functions and various signalling cascade. In *E.coli* transmemebrane PAS domain containing sensor kinase PhoQ and its associated partner PhoP acts as multiple function regulators that monitor different processes like Mg^{2+} transport, type III secretion , low pH sensing, acid stress resistance and cell wall structure remodelling. In low divalent metal ion concentrations, metal ions bind to the transmembrane PAS domain of PhoQ sensor kinase and as a consequence PhoQ activates PhoP [51, 97]. Pathogenic organisms adapt at low divalent cation environment as well as acidic pH by their PhoQ proteins [98]. In PhoQ sensor kinase, acidic cluster of residues in the PAS domain is essential for sensing the divalent metal ions and keep continuing the PhoQ sensor kinase in a repressed state [52] (Fig.15).

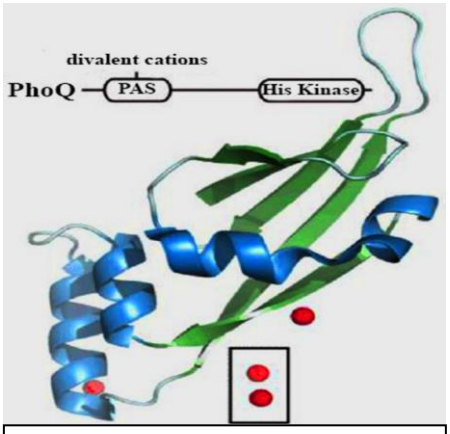

Fig. 15. Divalent cation binding PAS domain in PhoQ protein of *S. typhimurium* (PDB ID: 1YAX) (Adapted from Ref.34)

1.5.4.4. d) Role in nutrient sensing and metabolism

In aerobic metabolism, bacteria used carboxylic acids as a main source of carbon and energy [99-101]. Bacteria have evolved sensory systems that are required for uptake and subsequent metabolism of carboxylic acid compounds. DcuS and CitA are two important sensory histidine kinase which are able to sense C4 and C6 carboxylate containing substrates, this mechanism facilitates by extracytoplasmic PAS domain [53, 102-104]. After activation DcuS and CitA, they regulate the gene expression, which is involved in C4 and C6 utilization and transport [99, 105]. In rhizobia, dicarboxylate transportation is monitored by an additional sensory kinase named DctB, which are able to bind with C3 and C4

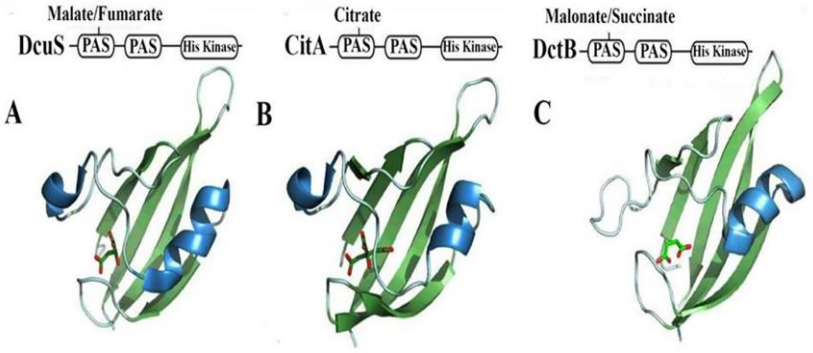

Fig. 16. A) Malate/fumarate binding PAS domain in DcuS protein of *E.coli* (PDB ID: 3BY8). B) Citrate binding PAS domain in CitA protein of *K.pneumoniae* (PDB ID: 1P0Z). C) malonate/succinate binding PAS domain in DctB protein of *S.meliloti* (PDB ID: 3E4O) (Adapted from Henry et al, Annu Rev Microbiol.,2011)

substrates in an identical region of the PAS domain, implying absolutely different residues as juxtaposed to DcuS and CitA [103, 106] (Fig. 16). So, it can be said that the PAS domain can bind to different carboxylic acids using numerous side chain interactions.

1.5.4.5. e) Role in visible light signal sensing

Photoactive yellow protein (PYP) from the purple sulphur bacteria *Halorhodospira halophila* was the earliest PAS domain containing protein whose crystal structure was resolved. As a result, PYP was contemplated as a model of PAS domain containing protein for a long period [70, 107]. The PYP have a single PAS domain which interacts with 4-hydroxycinnamic acid (p-coumaric acid – secondary metabolite) and covalently bound through a conserved cysteine residue [108, 109]. This covalent interaction facilitates blue/near UV light sensing [110]. PYP acts as negative regulator of phototaxis in halophilic bacteria *Halorhodospira halophile* [111]. Multiple domain containing proteins which encodes PYP domain has been located in different bacteria [112]. PPr is multi-domain protein from *Rhodospirillum contenum* which contains blue light absorbing PYP domain alongside with a red light absorbing bacteriophytochrome domain [113]. This PPr protein also helps in the development of starvation resistant cysts [114] and chemotaxis signalling pathway [115] (Fig.17).

1.5.4.6. f) Visible light sensing by flavin binding PAS domain

A new group of PAS domain is identified in plants that can bind with a flavin cofactor and acts regulators of phototrophic responses [116]. PAS domain bind with a flavin cofactor which leads to formation of a cysteinyl flavin covalent adduct which is responsible for blue light absorption [117]. The crystal structure of photosensory PAS domain is known as LOV domain [87, 118, 119]. The LOV domain contains conserved group of amino acids which creates a polar network of interactions along with isoalloxazine ring, ribotyl chain and phosphate of the flavin (**Fig.18**). This flavin binding consensus sequence and conserved cysteine residue has been used to anticipate putative LOV domain in other organisms. As a result, it is

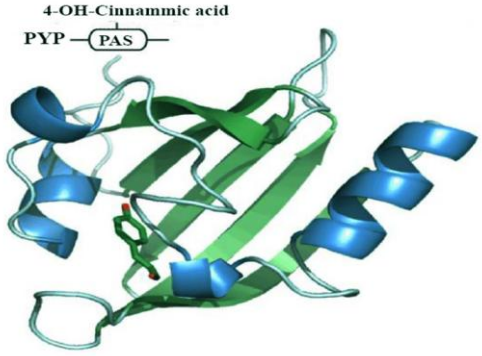

Fig.17. 4-hydroxy cinnamic acid binding PAS domain in PYP protein of *H. halophila* (PDB ID: 2PHY) (Adapted from Henry et al, Annu Rev Microbiol., 2011)

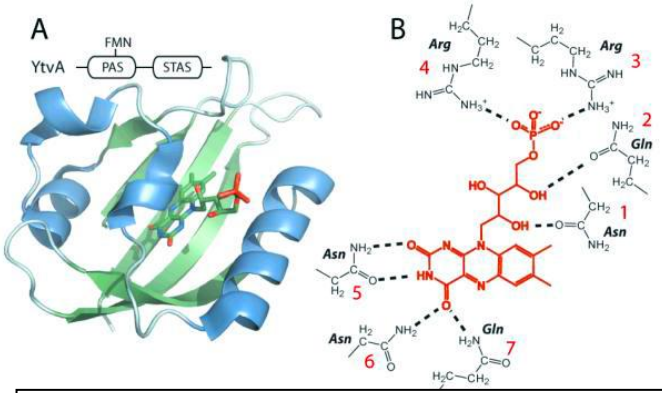

Fig.18. Flavin binding PAS domain in protein YtvA of *B. Subtilis* (PDB ID: 2PR5)(Adapted from Henry et al, Annu Rev Microbiol., 2011)

found that LOV domain is widely present in prokaryotes. In many bacterial species that encodes LOV domain but they are not photosynthetic, phototactic and not pigmented [45].

1.5.4.7. g) Role in redox potential sensing

Some PAS domain containing proteins are capable of sensing cellular redox potential through a bound flavin adenine dinucleotide (FAD) cofactor. The most well studied PAS domain containing protein is the transmembrane aerotaxis receptor (Aer) present in *E. coli* [56]. It controls the energy state of a cell by sensing the status of its electron transport [120, 121]. NifL and MmoS are two PAS domain containing proteins which are also capable of sensing redox potential [68, 122]. In Aer, NifL and MmoS the isoalloxazine ring moiety and phosphate of FAD is bound to conserved polar amino acids like asparagines and lysine/asparagine residues of the PAS domain (Fig.19). The PAS domain of Aer regulates the aerotaxis responses by controlling its interaction with CheA, a taxis kinase. In NifL, the FAD bound PAS domain monitors the expression of nitrogen fixation genes by interacting with DNA binding protein NifA in a redox dependent manner [123].

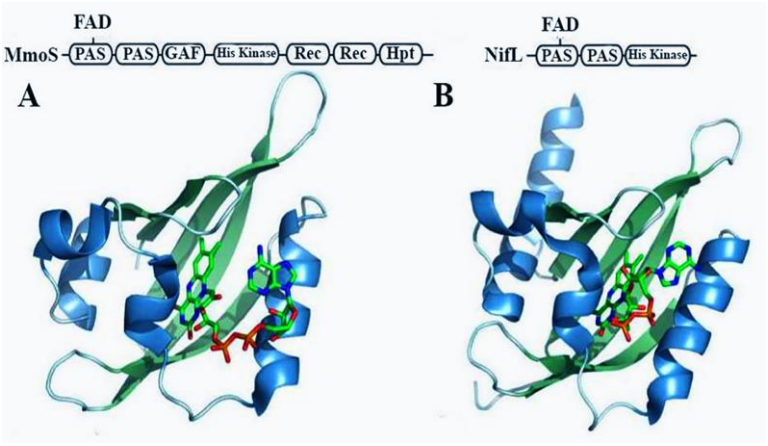

Fig. 19. FAD binding PAS domain. A) MmoS protein of *M. capsulatus* (PDB ID: 3EWK). B) NifL protein of *A. vinelandii* (PDB ID: 2GJ3)(Adapted from Henry et al, Annu Rev Microbiol., 2011).

1.6. Role of PAS domain containing protein in eukaryotes

1.6.1. a) Hypoxia response pathway

Most of the organisms are capable of sensing different oxygen concentration with different pathways. Mammalian cell respond to low oxygen (hypoxia) in various ways. They respond to hypoxia through modification of oxygen transport, glucose metabolism, angiogenesis and differentiation states [124-130]. The pathway responsible for hypoxia involves in the alteration of the expression of several genes by the hypoxia inducible transcriptional factor (HIF's) (Fig. 20). In early 1990, HIF complex was discovered while scrutinising the regulation of the erythropoietin hormone [131, 132]. HIF complex contained a pair of PAS domain containing proteins. HIFs are heterodimers which incorporates one alpha and one beta subunit [133]. Mammals contain three HIF-α genes which are HIF-1α, HIF-2α, HIF-3α [134-136]. The hypoxic reciprocation is an intricated pathway which is regulated at transcriptional level and as well as post-transcriptional level. In normoxic condition HIF proteins are expressed steadily, but they are swiftly

degraded through proteasomal complex. This degradation is moderated by proline residues. Rapid hydroxylation of proline residues occurred through three prolyl hydroxylase enzymes (Egln1, Egln2, Egln3) in normoxic condition [137]. This hydroxylation leads to ubiquitinylate of HIF by E3 ubiquitin ligase and subsequently proteasomal degradation of HIF is occurred [138, 139]. Under hypoxic condition (less than 2% oxygen level), prolyl hydroxylase does not work properly and as a consequence HIF protein level increases gradually and subsequently translocated to nucleus, where HIF complex formation occurred through PAS domain mediated interaction. HRE present in genes which encodes erythropoietin, prolyl-4-hydroxylase, chemokine receptor 4, Met protooncogene, Bcl2/adenovirus E1B-interacting protein 3, nitric oxide synthase 2-inducible, endothelin 1, transferin, heme oxygenase1 and others. These genes are responsible for survival of adapting cells, tissue and different organs under low oxygen condition [140-146]. Hif-1a, Hif-2a and Hif-3a perform various functions inside the cell. Hif-1a primarily controls the metabolic responses in hypoxic condition where as Hif-2a governs erythropoietin expression [147, 148]. In hypoxia response pathway PAS domain acts as a regulator of protein/protein interactions.

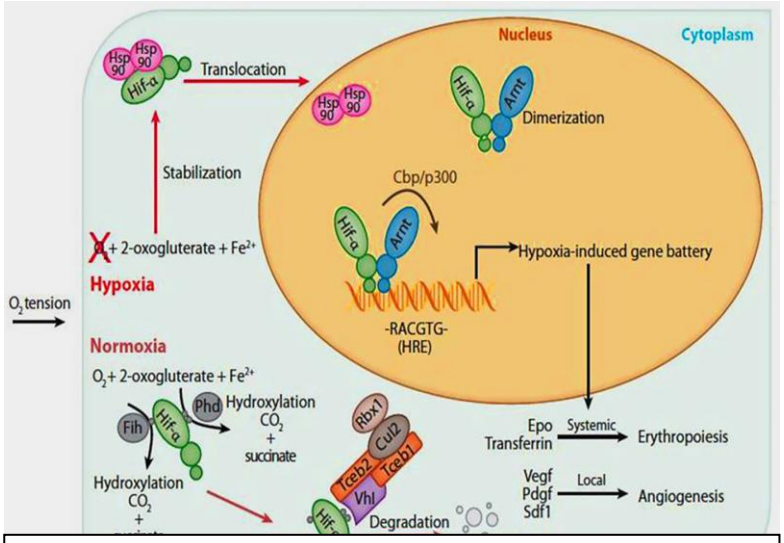

Fig. 20. Role of PAS protein in hypoxia response pathway (Adapted from McIntosh et al, Annu. Rev. Physiol., 2010)

1.6.2. b) Dioxin response pathway

Eukaryotic organisms respond to a numbers of small molecules in the environment, polycyclic aromatic hydrocarbon and polyhalogenated derivatives, the dioxins are produced from industrial processes, combustion and cigarette smoking [149-151]. These responses towards small molecule can be either adaptive or toxic. Dioxins are generally high affinity receptor agonists which are tough to metabolize. As a consequence, it triggers different harmful responses which include recalcitrant acne, promotion of tumour, wasting, thymic involution and death [149, 152]. Ahr, Ahrr, Arnt and Arnt2 take part in this pathway [153]. The PAS B domain of Ahr protein binds to ligands [153, 154] which tend to expose a nuclear localization signal, as a consequence phosphorylation occurred by protein kinase C (PKC) which leads Ahr to localize to the nucleus [155]. After localizing at nucleus, Ahr interacts with Arnt and form Ahr-Arnt complex, further

these complexes are able to bind with dioxin response element (DRE) present in its target genes [156]. Ahr-Arnt complex binds to the DRE of specific genes which facilitates the recruitment of RNA polymerase II in the complex which further leads to switch on the target genes. Ahr also harbours a cis acting nuclear export signal (NES) which switched off the Ahr target genes. This cis acting NES allows shuttling back of the Ahr to the cytoplasm [157] where it is degraded by proteasomal machinery [153, 158, 159]. Like hypoxia response pathway, the key functions of the PAS domain in the dioxin signalling pathway is protein-protein interactions. PAS-B domain present in Ahr is also able to bind with polyhalogenated aromatic hydrocarbons and polycyclic aromatics which functionally switched on them (Fig.21).

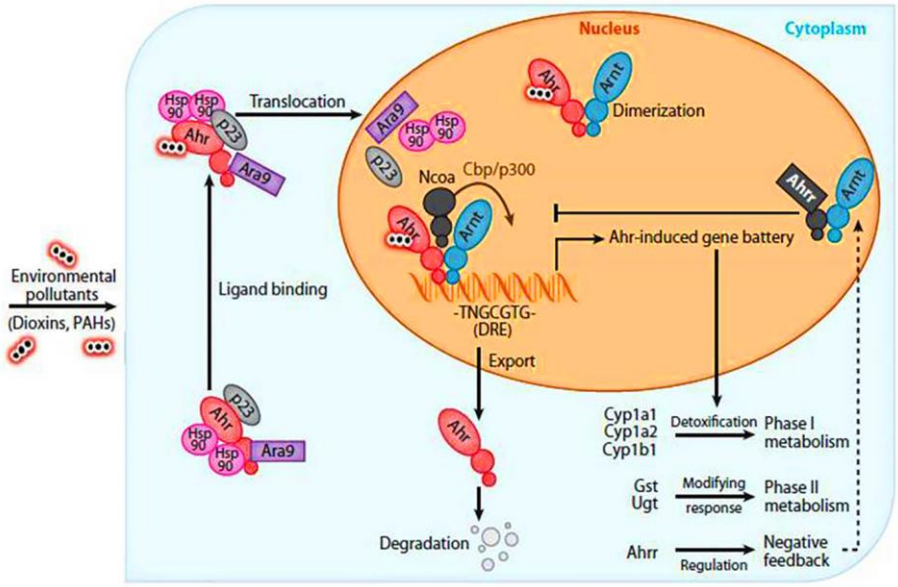

Fig. 21. Role of PAS protein in dioxin response pathway (Adapted from McIntosh et al, Annu. Rev. Physiol., 2010)

1.6.3. c) Circadian pathway

Most of the mammals behaviour, physiology is rhythimic nearly in a period length close to a day or 24 hours, a circa [160] diem (day) rhythm [161]. The circadian rhythm in human maintained by a master clock, existing ina suprachiasmatic nucleus of the hypothalamus which proceeds light related information from the eye [162]. Sleep and wake conditions also regulated by this circadiam rhythm. Different organ system of human like circulatory [163], digestive [164], endocrine [165], nervous [166] system has similar clock proteins. Most of those participating clock proteins are PAS domain containing protein. Bmal1/ Bmal2 and clock /NPAS2, these two proteins are the most important proteins which involves in the circadian signal transduction pathway. These two proteins are capable of interacting with several co-activators such as Kat2b, the CREB binding protein, Crebbp and EP300. This interaction makes a complex which facilitates recruitment of RNA polymerase II. As a consequence, it forms an activator complex which leads to activate transcription [167-170]. This activator complex regulates several genes which includes the repressor proteins of two distinct classes, one is the PAS domain containing Per1/Per2/Per3 family and the other is non PAS domain containing

cryptochromes1 and cryptochromes2 (cry1, cry2 family) [171-173]. While these repressor proteins assemble in the cytosol and form a complex which is then phosphorylated by two casein kinases, Csnk1e and Csnk1d. This phosphorylation is critical for modulating the nuclear localization and the activity of the complex [174, 175]. Along with protein-protein interactions, NPAS2 and Per2 can bind with heme [176, 177]. Per2 bound with heme is more stable and escalate its potency to modulate Clock-Bmal1. In contrast, heme binding to NPAS2 permits nitric oxide (NO) sensing. Thus PAS domain engaged in the circadian pathway through protein-protein interaction together with ligand binding to moderately switching the cellular operations (**Fig. 22**).

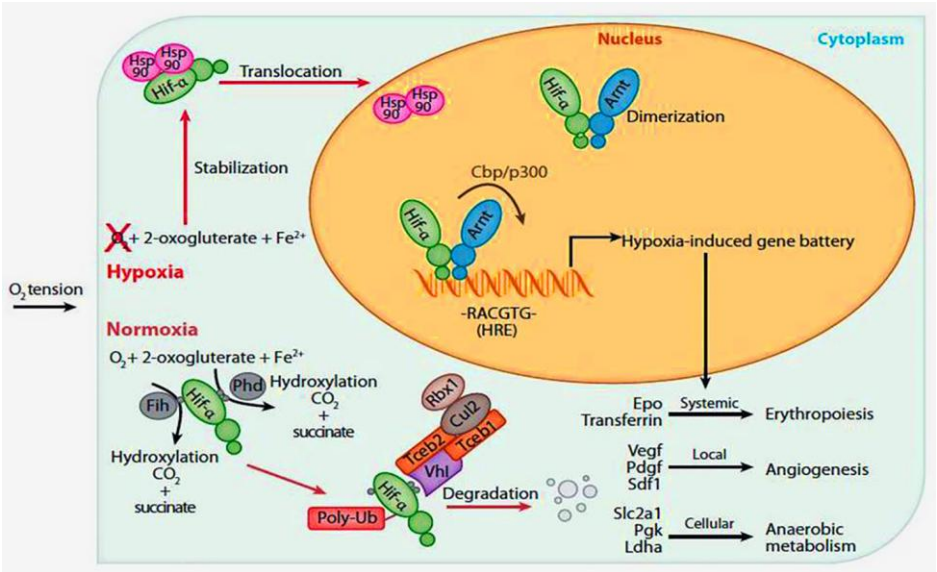

Fig.22. Role of PAS protein in circadian pathway (Adapted from McIntosh et al, Annu. Rev. Physiol., 2010)

Materials and Methods

MATERIALS & METHODS

2.1 Materials

2.1.1. Kit

Name of kit	Purchased from
Genomic DNA isolation kit	Qiagen and Thermo
Plasmid DNA isolation kit	Qiagen and Thermo
DNA extraction kit from agarose gel	Qiagen and Thermo
PCR product purification kit	Qiagen
Ni-NTA agarose resin	Qiagen

2.1.2.Media

Culture media	Purchased from
Parasite culturing media- M199	Gibco
RPMI 1640	Gibco
Schneider's Drosophila media	Gibco
fetal bovine serum	Gibco
Luria Bertani (LB) medium	HiMedia
Terrific Broth (TB)	HiMedia
Yeast extract, Tryptone	HiMedia

2.1.3. Molecular biology marker and reagents

Marker and reagents	Purchased from
Prestained protein molecular weight marker	Thermo Scientific
Unstained protein molecular weight marker	Thermo Scientific
DNA molecular weight marker	Thermo Scientific
Restriction enzymes	Thermo Scientific
T4 DNA ligase	Thermo Scientific
High fidelity DNA polymerase	Thermo Scientific
Taq DNA polymerase	Thermo Scientific

2.1.4. Antibody

Name of antibody	Purchased from
Anti α-tubulin antibody	Cell Signaling
Fluorescent tagged secondary antibody	Thermo Scientific
Alkaline phosphatase conjugated secondary antibody	Sigma

2.1.5. Antibiotics and Inhibitors

Antibiotics and inhibitors	Purchased from
Penicillin-streptomycin solution	Thermo Scientific
Ampicillin	SRL
Kanamycin	SRL
Tetracyclin	SIGMA
Streptomycin	SRL
Protease inhibitor cocktail	Roche
G-418 SOLUTION	Roche
NBT/BCIP SOLUTION	Amresco
PMSF()	SRL
RNaseA	Thermo Scientific
Proteinase K	Thermo Scientific

2.1.6. Membrane

Name of Membrane	Purchased from
PVDF	Merck Millipore
Nitrocellulose Membrane	Merck Millipore

2.1.7. Other Reagents and Chemicals

Reagents and chemicals	Purchased from
TEMED	Sigma
β-mercaptoethanol	Sigma
Bromophenol blue	Sigma
Coomassie brilliant blue R-250	Sigma
SDS	Sigma
Tris	Sigma
Glycine	Sigma
EDTA	Sigma
Lysozyme	Sigma
γ-globulin free BSA	Sigma
DMSO	Sigma
Ethidium bromide	Sigma
Propidium Iodide	Calbiochem
Protein estimation reagent	Bio-Rad Laboratories
Acrylamide-bis acrylamide solution	HiMedia

Glacial acetic acid, glycerol, KCl, NaCl, KOH, NaOH, KH_2PO_4, K_2HPO_4, NaH_2PO_4, Na_2HPO_4, were purchased locally either from Qualigens or SRL. Yeast extract, peptone, tryptone, agar-agar, acrylamide-bis acrylamide solution and other bacterial growth media

components were the products of HiMedia. Methanol, ethanol was purchased from Merck.

2.1.8 Strains used

A) *Leishmania*

Leishmaniamajor (5 ASKH)

B) Macrophage

RAW 246.7

C) Bacterial strain

The following strains of *Escherichia coli* were used:

DH5α, JM109, BL21 (DE3) pLyS

2.1.9. Plasmid vectors used

For cloning of open reading frame: pET15B.

2.1.10. *Leishmania* culture media

M199 powder	11.0 gm
HEPES	5.1 gm
Adenine	200 μM
Folic acid	150 μg/ml
Haemin	10 μg/ml

Volume was made up to 1000 ml and pH adjusted to 7.4 with HCl. Penicillin-streptomycin was added at 1.5% and gentamycin added to 50μg/ml. Finally, the media was filtered through 0.22 μM sterilized filter unit and keep it room temperature overnight for checking any contamination occurred or not, finally media was stored at 4°C.

2.1.11. Bacteria culture media

A. Luria Bertani(LB) medium, (pH – 7.5)

Bacto-tryptone	10g/L
Yeast extract	5g/L
Sodium chloride	10g/L

B. Terrific Broth (TB) (pH 7.5)

Bacto- tryptone	12g/L
Yeast extracts	24g/L
Glycerol	4ml/L

2.2. Buffers and solutions

A. Potassium phosphate buffer

KH_2PO4	170 mM

K_2HPO4	720 mM

B. Phosphate buffered saline (pH– 7.4):

Na_2HPO_4	14.4 g/L
KH_2PO_4	2.4 g/L
NaCl	80 g/L
KCl	2.0 g/L

C. 50X TAE buffer

Tris base	242 g/L
Glacial acetic acid	57.1 ml/L
EDTA (0.5 M, pH – 8)	100 ml/L

D. Electroporation buffer for *Leishmania major*

HEPES (pH- 7.4)	21 mM
NaCl	137 mM
NaH_2PO_4	0.7 mM
D-glucose	6 mM

E. SDS-PAGE running buffer

Glycine	14.4 g/L
Tris	3.0 g/L
SDS	1.0 g/L

F. Bacterial cell lysis buffer

Tris-HCl, pH-7.5	50 mM
NaCl	150 mM
PMSF	1 mM
B-mercaptoethanol	0.05%
Lysozyme	1 mg /ml
Protease inhibitor (without EDTA)	0.5 tablet

G. Equilibration buffer for protein purification

Tris-HCl, pH-7.5	50 mM
NaCl	250 mM
PMSF	1 mM
Imidazole, pH-7.5	20 mM
Glycerol	5 %

H. Buffer for protein elution

Tris-HCl, pH-7.5	50 mM
NaCl	250 mM
PMSF	1 mM
Imidazole, pH-7.5	20 mM
Glycerol	10%
Protease inhibitor (without EDTA)	0.5 tablet

2.3 Oligonucleotides used

Primer	Primer sequence (Restriction sites are underlined)	Purpose
Primer1	5'AAAAGGATCCATGTATCAGGACTCGAAGAT 3'	Cloning of
Primer2	5' AAAAAAGCTTCTACAGCTTAGGAGAGGC 3'	LmPAS-PGK
Primer3	5'AAAAGGATCCGCCGCCCTCACCCCGAA 3'	Cloning of Δ115
Primer4	5' AAAAAAGCTTCTACAGCTTAGGAGAGGC 3'	LmPAS-PGK
Primer5	5' CGTCCCCACTGCCCCGCTGGATGTACAGG 3'	Cloning of H57A
Primer6	5'CCTGTACATCCAGCGGGGCAGTGGGGACG 3'	
Primer7	5' CCTTATGGCAGCCCTTGCCAAC 3'	Cloning of H71A
Primer8	5' GTTGGCAAGGGCTGCCATAAGG 3'	
Primer9	5' CCGCGACCCCGCCGCAACAGTGG 3'	Cloning of H89A
Primer10	5' CCACTGTTGCGGCGGGGTCGCGG 3'	
Primer11	5' GTTCGGGGTGGCCATCAACATCCG 3'	Cloning of
Primer12	5' CGGATGTTGATGGCCACCCCGA 3'	H111A

2.4. Methods

2.4.1. Methods for cloning LmPAS-PGK and Δ115 LmPAS-PGK

2.4.1.1. Isolation of genomic DNA from *Leishmania major*

The genomic DNA from *Leishmania major* was isolated by using the 'Genomic DNA isolation kit' (Qiagen). 2×10^8*Leishmania major* promastigote cells were washed twice with 1X cold PBS and pelleted down at 4000 rpm for 6 mins in a 1.5 ml micro-centrifuge tube. 200 µl Buffer ATL (lysis buffer), containing 20 µl proteinase K and 10 mg/ml RNaseA was added and pulse vortexed for 30 secs. It was then incubated at 56°C until the cells were completely lysed (15 mins approximately). After complete cell lysis, 200 µl of ethanol was added and again pulse vortexed followed by a pulse spin. Then the mixture was transferred on to a spin column provided by the manufacturer. It was centrifuged at 8000 rpm for 1 min. The solution deposited at the bottom tube was discarded. 500 µl of AW1 buffer was then added to the column and centrifuged at 8000 rpm for 1 min. The solution deposited at the bottom of tube was again discarded. Then,

500 µl of AW2 buffer was added to the column and centrifuged at 14000 rpm for 3 mins. The supernatant deposited at the bottom of tube was discarded. Finally, DNA was collected in a fresh centrifuge tube by eluting the column with 75 µl of nuclease free water by centrifuging at 14000 rpm for 1 min. For long-term storage of DNA was stored at –20°C. The DNA purity was checked with an A_{260}/A_{280} ratio of 1.7–1.9.

2.4.1.2. Polymerase Chain Reaction (PCR)

PCR amplification was done by using purified leishmanial genomic DNA as template. Each 50 µl reaction having 30 ng of template genomicDNA, 20 picomoles of forward and reverse primers, 200 µM of each dNTP, 1.5 mM $MgCl_2$ and 2.0 U of High fidelity DNA polymerase. PCR was done in a Veriti Thermal Cycle (Applied Biosystems) for 30 cycles. Each cycle consisted of: i) denaturation at 95°C for 1.0 minutes; ii) annealing at 52°C for 30 seconds; iii) extension at 68°C for 2.5 minutes and iv) a final cycle of extension at 68°C for 10 minutes. The amplified product was purified by PCR purification kit (Qiagen).

2.4.1.3. Cloning of LmPAS-PGK andΔ115LmPAS-PGK from genomic DNA

The full length LmPAS-PGK and Δ115LmPAS-PGK (deleted 115 amino acid residues from N-terminal) were amplified by using the gDNA (30ng/µl) as the template. Primer1 and primer 2 were used for full length LmPAS-PGK. Primer 3 and primer 4 were used for Δ115LmPAS-PGK. The amplified full length LmPAS-PGK product was cloned into the HindIII and BamHI sites of the prokaryotic expression vector pTrcHisA whereas the truncated Δ115LmPAS-PGK was cloned into the HindIII and BamHI sites of the vector pET28a.

2.2.1.4. Agarose gel electrophoresis

1% agarose gel was run depending upon the size of DNA fragments to be separated. DNA samples were mixed with 1X DNA loading buffer (Thermo Scientific) and then loaded onto the wells of the submerged gel. The gel was run in 1X TAE at a constant voltage of 12 V/cm. DNA was visualized by staining with ethidium bromide solution (0.5 µg / ml) by UV irradiation.

2.2.1.5. Isolation of plasmid DNA

10 ml of bacterial cell culture containing the plasmid of interest, was grown overnight in LB medium and then harvested in a 2.0 ml micro centrifuge tube. 300 µl of resuspension buffer P1 containing RNaseA was added to the bacterial cell pellet and the cells were vortexed mildly. 300 µl of lysis buffer P2 was added to that solution and mixed by gentle shaking. 400 µl of neutralizing buffer N3 was finally added and was mixed gently. The resultant solution was centrifuged at 14000 rpm for 30 minutes to eliminate the cell debris, genomic DNA and proteins. The supernatant was then loaded on to the column

provided by the manufacturer and centrifuged at 14000 rpm for 1 minute. Solution deposited at the bottom tube was discarded. Column was then washed with 500 µl of binding buffer PB followed by a wash with 750 µl of wash buffer PE containing ethanol. Then the empty column was re-centrifuged to get rid of any trace of PE buffer. Finally, 70 µl of autoclaved double distilled water was added to the column and it was placed on the top of a fresh 1.5 ml micro centrifuge tube. After centrifugation DNA pellet (70 µl) was deposited in the micro centrifuge tube. Concentration of DNA was checked spectrophotometrically or by running agarose gel with suitable standards.

2.4.1.6. Restriction digestion of the amplified DNA and the cloning vector

Restriction digestion of the PCR amplified DNA and the cloning vector was carried out at 37°C for 4 hrs according to the following

Name of reagents	Volume
Sterile, deionized water	9.5 µl
Restriction Enzyme 10X Buffer	4.0 µl
Acetylated BSA, (10 µg/µl)	0.5 µl
DNA, (1 µg/µl)	25 µl
Restriction Enzyme, (10 u/µl)	1 µl
Final volume	40 µl

2.4.1.7. Purification of DNA by gel extraction kit

The digested products were run on a 1% agarose gel from where the DNA band of interest, as visualized, was cut out by using a sterile scalpel blade. The gel piece obtained was taken in a micro centrifuge tube. The DNA was eluted out of the gel piece by using gel extraction kit (Qiagen): Briefly, QG buffer (300 µl/100 mg gel piece) was added to the gel piece and was incubated for 10 minutes at 56°C and vortexed at every 2 minutes intervals. After complete solubilization of the gel, the suspension was loaded onto a spin column provided by the manufacturer. It was centrifuged at 14000 rpm for 60 seconds and the solution at the bottom tube was discarded. 500 µl of QG buffer was added to silica column and again centrifuged at 14000 rpm for 60 seconds. Like before, the supernatant deposited at the bottom tube was discarded. Pellet was resuspended in nucleic acid binding buffer, PB (500 µl) and again centrifuged. Then the pellet was washed twice with wash buffer PE containing ethanol and centrifuged at 14000 rpm for 60 seconds. The column was re-centrifuged to eliminate any traces of PB buffer. 35 µl of autoclaved double distilled water was then added to the pellet, incubated at room temperature for 2 mins. Finally, it was centrifuged at 14000 rpm for 1 minute to obtain purified DNA.

2.4.1.8. Ligation of insert and vector DNA

Ligation reactions were performed at 22ºC for 1 hour in the following way using 'rapid DNA ligation kit' from 'Thermo Scientfic'

Name of reagents	Volume
Vector DNA (1 µg/µl)	0.1 µl
Insert DNA (0.4 µg/µl)	1.0 µl
5X buffer	3.0 µl
T4 Ligase (10U/µl)	1.0 µl
Ionised water	5.9 µl
Final volume	10 µl

2.4.1.9. Transformation

The transformation of *E.coli* cells with purified plasmid DNA or ligation mix was carried out by the conventional $CaCl_2$ heat shock method. Desired amount of DNA was mixed with 100 µl of *E.coli* competent cells (previously prepared). It was then kept in ice for 30 mins. Next, heat shock was given for 90 secs at 42ºC and then immediately put in ice for 5 mins. 1 ml of LB media (without antibiotic) was added to the tube and incubated for 1 hour at 37ºC. After 1 hour, it was centrifuged at 14000 rpm for 2 mins and the pellet was dissolved in the remaining media. The resuspended pellet was then spread plated on a 90 mm bacterial culture petriplate having the concerned antibiotic selection.

2.4.1.10. DNA sequencing

Automated DNA sequencing was done according to the chain termination method. Briefly, a 10µl reaction was prepared containing 1 µl BD mix (provided with the sequencing kit), 1.5 µl of 5X buffer, 150-200 ng of template DNA and 6 pmol of sequencing primer. PCR was carried out in a Perkin Elmer automated thermal cycler for 30 cycles. Each cycle consisted of: i) denaturation at 96°C for 10 sec; ii) annealing at 53°C for 10 sec; iii) extension at 60°C for 4 minutes. After the PCR,20 µl of water was added to the reaction mixture. The DNA was purified using ethanol precipitation method. 19 µl high dye (provided with the kit) was added to the DNA pellet and kept in dark for 15 mins. The mixture was then heat-chilled and finally 10 µl of the mix was loaded on to a well of 96-well plate of Perkin Elmer ABI Prism™ DNA sequencer.

2.5. Methods for expression & purificationof LmPAS-PGK and Δ115LmPAS-PGK

2.5.1. Expression and purification

In order to express the recombinant full length LmPAS-PGK and the truncated (Δ115LmPAS-PGK), the respective plasmids were transformed separately into competent *E.coli*BL21 (DE3) pLys cells. For the full length LmPAS-PGK, the transformed cells were grown in 50 ml Luria-Bertani (LB) broth containing 200 µg/ml ampicillin at 37°C. The culture which grew overnight was inoculated in 500 ml of modified terrific broth

(TB), incubated at 37°C for 2hrs and then induced with 0.5 mM IPTG.*E. coli* cells grown at 25°C and 180 rpm for 20 hrs, were then pelleted and resuspended in 50 mM Tris-HCl buffer pH 7.5 containing 0.5 mM EDTA, 10% glycerol, 150 mM NaCl, protease inhibitor cocktail (Roche), 0.5 mM PMSF and 1.0 mg/ml lysozyme. The resuspended solution was kept for 45 mins at 4°C and then the cells were lysed by sonication. The lysate was centrifuged at 14000 rpm for 1 hr. The supernatant was loaded on to a Ni^{2+}-nitrilotriacetate column. After loading the crude extract, the column was washed with 10 column volumes of washing buffer (50 mM Tris-HCl pH 7.5, 150mM NaCl, 10% glycerol and 1 mM PMSF). The enzyme was eluted with the washing buffer supplemented with 250 mM imidazole. The ezyme thus obtained was dialysed three times against 50 mM Tris-HCl buffer pH 7.5, 150 mM NaCl, and 10% glycerol. Purification of Δ115LmPAS-PGK catalytic domain was carried out in the same way as above except the cells were grown in media containing 50 µg/ml of kanamycin. Molecular weight and purity of the proteins were confirmed by 13% SDS PAGE.

2.5.2. SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed using the method of Laemmli[177]. Briefly, induced cells or enzyme preparations were boiled for 10 minutes with Laemmli buffer and loaded onto either 10% or 13% cross-linked SDS-polyacrylamide gel.

2.5.3. SDS-gel staining by Coomassie Brilliant Blue

The SDS-PAGE gel was immersed in a solution of acetic acid (10%) and methanol (45%) containing 0.25% Coomassie brilliant blue R-250 for the purpose of staining. Staining was usually done by keeping the gels for 1-2 hrs as per requirement. Destaining was carried out with the destaining solution containing acetic acid (10%) and methanol (45%).

2.6. Methods for physical characterization

2.6.1. Protein Concentration Determination

Protein concentrations of the fulllength LmPAS-PGK, the truncated Δ115LmPAS-PGK and all mutant proteins were measured using Bio-Rad reagent. BSA was used as the standard [178].

2.6.2. Enzyme assay

The kinetic studies for LmPAS-PGK, Δ115LmPAS-PGK and all mutant proteins were carried out at 25°C on Shimadzu UV-2550 spectrophotometer using quartz cuvette of 1.0-cm path length. The composition of the assay mixture was 40 mM Tris buffer (pH 7.5) (Amresco), 0.15 mM NADH (Merck), 0.8 mM $MgSO_4$, 1 mM ATP (Sigma), 2.5 mM 3-phosphoglyceric acid (Sigma) and 0.04U of rabbit muscle glyceraldehyde 3 phosphate dehydrogenase (Sigma). After incubating for 5 mins to achieve the temperature

equilibrium, the reaction was started by adding the pure enzyme. Absorbance at 340 nm from 0 to 5 mins was recorded. The different PAS domain ligands were individually added in the assay mixture at their effective concentration.

2.6.3. Size exclusion chromatography

Both the full length LmPAS-PGK and the truncated Δ115LmPAS-PGK was analyzed by size exclusion chromatography at 28°C using a column namely Biosuite™ 250 (Waters) in an HPLC system (Waters) pre-equilibrated with potassium phosphate buffer (50mM, pH 7.5)containing NaCl (150mM), with a flow rate 0.8 ml/min at A_{280}.The column was calibratedwith a standard protein mixture containing glutamate dehydrogenase (290 kDa), lactate dehydrogenase (142 kDa), enolase (67 kDa), myokinase (32 kDa) and cytochrome c (12 kDa).

2.6.4. Determination of the pH dependent enzyme activity

The pH-optimum of LmPAS-PGK and Δ115 LmPAS-PGK was determined by measuring kinetic properties of both the proteins using the following buffers (50 mM): acetate (pH 3.5.0-5.5), MES and phosphate buffers (pH 5.5-6.5), Tris-HCl (pH 6.8-8.8), TEA-HCl (pH-7.5) under standard conditions. The pH was adjusted at room temperature.Shimadzu UV-2550 spectrophotometer with quartz cuvette of 1.0-cm path length was used.

2.6.5. Fluorescence quenching

In order to determine how Mg^{2+} binding alters the folding properties of full length LmPAS-PGK, Δ115 LmPAS-PGK and mutant proteins fluorescence quenching of the tryptophan residue were observed. A scan from 300 nm to 400 nm was recorded with a slit width of 5nm after exciting the protein at 295 nm using buffers of different pH. F-7000 FL Spectrophotometer, Hitachi was used with 1.0 cm path length cuvette. In order to measure enzyme–metal ion interactions, recombinant wild-type and H57A LmPAS-PGK enzyme at20 mM were allowed to equilibrate in 50 mM Tris/HCl buffer (pH 7.5) for 20 min before being titrated with divalent cation solutions. Wild-type and mutant were tested with increasing concentrations of $MgCl_2$ from 0 to 6 mM. The background fluorescence quenching caused by the dilution with the buffer was monitored by running parallel buffer control titrations. Data from three independent experiments were analyzed using nonlinear regression with 'One Site-Specific Binding' model (Y = Bmax*X/(KD + X) where Y is the fluorescence intensity, Bmax is the maximum specific binding, X is the ligand concentration, and K_D is the apparent equilibrium dissociation constant) in GRAPHPAD PRISM 6.0.

2.6.6. Isothermal Calorimetry

To determine the divalent metal binding parameters of theLmPAS-PGK enzyme, experiments using ITC were performed, and titration experiments were conducted using a

MicroCal VP-ITC Microcalorimeter (Presently Malverninstrument, Malvern, UK). The experiments were performed at 25 °C, and all samples were thoroughly degassed under vacuum for 30 min before loading. Solutions of $MgCl_2$ and the LmPAS-PGK were prepared in 50 mM Tris/HCl buffer at pH 7.5. The sample cell was loaded with 50 mM LmPAS-PGK solution, and the syringe was filled with a 10 mM (for wild-type) or 100 mM (for H57A) solution of the appropriate magnesium chloride in the same buffer. The $MgCl_2$ solution was added sequentially by multiple injections of a 7 µl (for wild-type) or 5 µl (forH57A) volume, at 180-s intervals with a stirring speed of 307 r.p.m. This experiment allows the determination of metal's heat of dilution in a buffer solution, which permits the correct determination of the heat exchange profile. ORIGIN 7.0 software (Northampton, MA, USA) was used for data acquisition and analysis. ITC data provided K, the binding constant; ΔH, enthalpy change; and ΔS, the entropy change values. Each experiment was repeated thrice, and the error value reflects the standard deviations.

2.6.7. Molecular modelling

Molecular modeling was used to build a structural modelfrom the LmPAS-PGK sequence. BLASTp using PDB allowed the selection of the ternary complex of PGK from *T. brucei* (PDB ID: 13PK) and the PAS domain of *S. Aureus* WalK protein (PDB ID: 4MN5) as the template for molecular modeling. Sequence alignment with two templates helped to get the full-length model structure. MODELLER9.1 produced five models, from which the best model, with the lowest energy, was selected.

2.6.8. Statistical analysis

All experimental data are expressed as the mean ± SD from at least three independent experiments. All the data are statistically analysed by analysis of variance (ANOVA) or Student's t test, wherever applicable using Origin 6.0 software (Microcal Software, Northampton, MA, USA). ANOVA was done by multiple comparison t-test (post hoc analysis) to evaluate the difference between individual groups. A p-value of less than 0.05 was considered statistically significant.

Results

CHAPTER 1

"Higher concentration of ATP and ADP acts as inhibitor"

“Higher concentration of ATP and ADPacts as inhibitor”

3.1.1. Background

3.1.2. Expression and purification of full length and truncated LmPAS-PGK

3.1.3. Size exclusion chromatography
- 3.1.3.1. I) Wild type LmPAS-PGK
- 3.1.3.2. II) PAS domain deleted LmPAS-PGK

3.1.4. ATP and ADP dependent LmPAS-PGK activity at pH 5.5

3.1.5. ATP and ADP dependent LmPAS-PGK activity at pH 7.5

3.1.6. Higher concentration of ATP and ADP acts as inhibitor

3.1.7. Discussion

3.1.1. Background

Like all other kinase enzymes phosphoglycerate kinase is transferase, which is involved in the catalyzation of reversible transfer of a phosphate moiety from 1,3 bis phosphoglycerate to ADP, as a consequence, it produces ATP and 3-Phosphoglycerate. PGK is involved in the first ATP producing step in glycolysis. ATP, ADP and PGK all are essential for maintaining cellular function under differential environmental condition like different pH, different nutritional stress condition and various divalent cations concentrations. Numourous literatures suggest that ATP and ADP switch is crucial for signalling cascade [178-180].

Three isoforms of PGK (PAS-PGK, PGKB, and PGKC) are present in human pathogen *Leishmania* spp [43, 44, 181, 182]. PGKB and PGKC enzymes are translocated to the cytosol and the glycosome (single membrane peroxisome like organelle), respectively. They are expressed in both stages of life cycles including the amastigote and the promastigote forms [44]. On the other hand, PAS-PGK has an N-terminal regulatory PAS domain and C-terminal catalytic PGK domain. It is present in the glycosome as well as the lysosome [182]. PAS-PGK is a monomeric in nature with 59-kDa mass. Comparative studies among overexpression, gene knockout and complement cell line demonstrate that LmPAS-PGK plays a crucial role in cell survival through autophagy. Human PGK, yeast PGK and leishmanial PGKs (B and C) show the optimum activities at neutral pH whereas the optimum activity of LmPAS-PGK is at pH 5.5. The comparative study between full length and PAS domain deleted enzyme suggest that the PAS domain of LmPAS-PGK primarily regulates the PGK catalytic activity with respect to pH changes but the mechanism of regulation by PAS domain at the molecular level is unknown.

Yeast 3-phosphoglycerate kinase is inactivated when it is incubated with pyridoxal 5'-diphospho-5'-adenosine which is a ATP analog [183]. The substrate ATP affords substantial protection against inactivation where another substrate 3-phosphoglycerate helps partially [183]. Yeast phosphoglycerate kinase is inactivated by its product [184]. ADP is a non-competitive inhibitor of MgATP and MgADP is competitive inhibitor of MgATP [184]. Hexokinase from *Trypanosoma brucei* is very sensitive to its substrate ADP or ATP at acidic pH, glycerol 3-P or glycerol 2-P protects the hexokinase from the inactivation by the substrate ATP or ADP at acidic pH [185]. To explore, whether the PAS domain of LmPAS-PGK helps to protect the PGK catalytic activity from the inactivation by the substrate ATP or ADP at acidic pH 5.5. This chapter has comparative studies between the substrate (ATP) / the product (ADP)-dependent PGK activity of full-length and PAS domain- truncated (Δ115) enzymes at various pH values.

3.1.2. Expression and purification of full length and truncated LmPAS-PGK

In order to addressing the biochemical and biophysical characteristics of LmPAS-PGK , both the wild type LmPAS-PGK and PAS domain deleted protein were expressed in *E.*

coli. Recombinant LmPAS-PGK and Δ115 LmPAS-PGK carrying a N-terminal six-histidine tag were purified using Ni^{2+}-NTA column. Purified LmPAS-PGK and PAS domain deleted LmPAS-PGK proteinsrun into SDS-PAGEat positions of 60 kDa (Fig.23, lane 4) and 45 kDa protein (Fig.23, lane 8), respectively, as expected from the theoretical relative molecular mass.

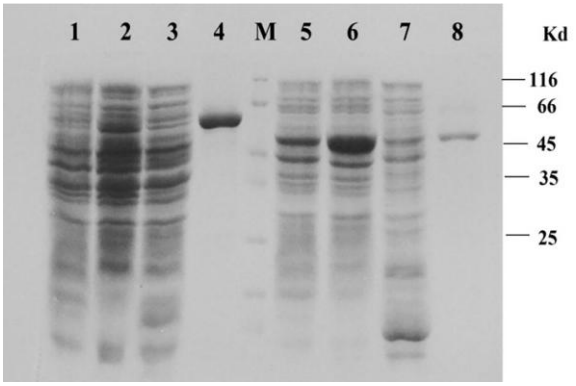

Fig.23. Purification of recombinant *L. major* full length PAS-PGK and Δ115LmPAS-PGK protein shown in a Coomassie Blue stained 13% SDS-PAGE analysis of protein fractions SDS-PAGE; Lane 1: -IPTG (full length), Lane 2: +IPTG (full length), Lane 3: lysate (full length), Lane 4: purified full length protein, Lane 5: Molecular weight Marker, Lane 6: -IPTG (Δ115), Lane 7: +IPTG (Δ115), Lane 8: Lysate (Δ115), Lane 9: purified (Δ115).

3.1.3. Size exclusion chromatography

3.1.3.1.Full length LmPAS-PGK

In order to find out the native state molecular mass of LmPAS-PGK, HPLC was done with purified wild type LmPAS-PGK protein. A single sharp peak was obtained around 60 kDa which indicates that the protein is monomeric in nature (Fig.24).

3.1.3.2. PAS domain deleted LmPAS-PGK

To detect, PAS domain have any role in changing proteins native structure. PAS domain deleted LmPAS-PGK was expressed and purified. Purified PAS domain deleted LmPAS-PGK was subjected to size exclusion chromatography using HPLC. A single sharp peak was obtained around 45 kDa for the truncated protein, indicating this protein is monomeric (Fig.25).

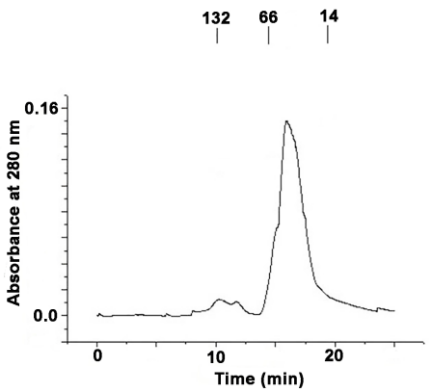

Fig.24. Size exclusion chromatography of wild type protein. (Reference to the standards, albumin -66kDa and lysosyme-14 kDa)

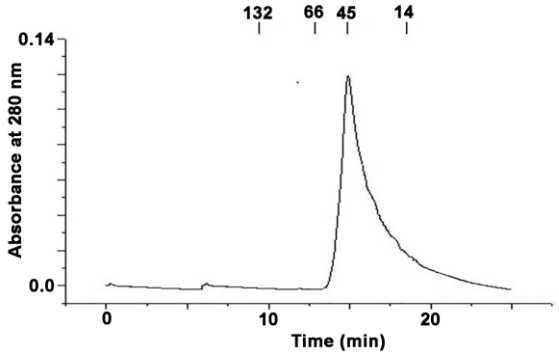

Fig.25. Size exclusion chromatography of PAS domain deleted protein. (Reference to the standards, albumin -66kDa, ovalbumin-45 kDa and lysosyme-14 kDa)

3.1.4. ATP and ADP dependent LmPAS-PGK activity at pH 5.5

It is known that hexokinase activity in *Trypanosome brucei* distinctly responsive to its substrate ATP or ADP at lower pH. Glycerol 3-P or glycerol 2-P regulated hexokinase

activity at low pH by intercepting substrate and product inhibition by ATP and ADP[185]. In order to find out, in case the PAS domain containing LmPAS-PGK secures PGK activity out from the deactivation by the ATP or ADP at low pH. We have tried to find out the comparative studies between the catalytic activity of full-length LmPAS-PGK and truncated(PAS domain-deleted) enzymes at different pH in substrate (ATP) and product (ADP) dependent activity (Fig. 26). Full-length enzyme exhibited saturation

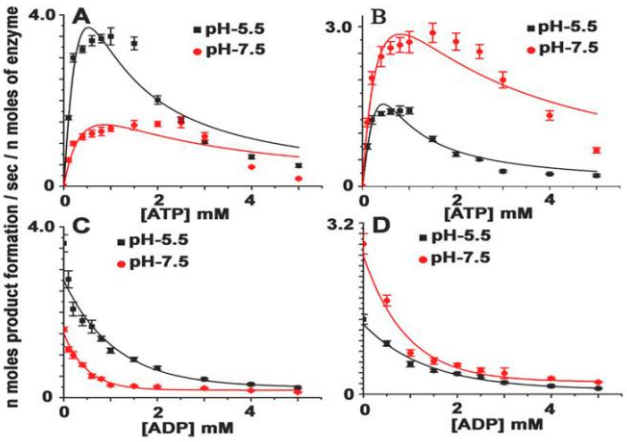

Fig. 26.Comparative studies of ATP- and ADP-dependent PGK activity between full-length and PAS domain-deleted LmPAS-PGK. Panels A and B show ATP-dependent PGK activity of wild-type and truncated enzymes, respectively. Panels C and D show ADP-dependent PGK activity of wild-type and truncated enzymes.

kinetics up to 1mM ATP (substrate) at pH 5.5 whereas the enzyme activity is inhibited at very high ATP concentration at same pH. Similar effect was observed in case of the PAS domain deleted enzyme at pH 5.5. Change in activity during different substrate(ATP) concentration was measured by using substrate inhibition model equation [Y = Vmax*X/(Km + X*(1 + X/Ki))] in software PRISM 6. Using this model produced the values of Vmax (maximum enzyme velocity), Km (Michaelis–Menten constant), and Ki (dissociation constant for substrate binding in a way that two substrates can bind to an enzyme) values of each full length and PAS domain-deleted (truncated) enzymes at acidic pH values. Vmax, Km, and Ki measurements of two enzymes full-length and truncated at pH 5.5 are tabulated in Table 12.

Table 12. Kinetic activity Measurements at pH 5.5.

Apparent rate constants from substrate and product inhibition model At pH 5.5				
enzyme	V_{max} (S^{-1})	k_m (mM)	k_i (mM)	ADP IC 50 (mM)
Full length	18 ± 5	1.0 ± 0.4	0.26 ± 0.16	1.1 ± 0.06
Truncated (Δ 115)	5.8 ± 1.0	0.41 ± 0.1	0.44 ± 0.1	1.3 ± 0.1

3.1.5. ATP and ADP dependent LmPAS-PGK activity at pH 7.5

ATP and ADP dependent LmPAS-PGK activity in pH 7.5 is very crucial because all the other PGK show their optimum activity at pH 7.5. But, in contrast PAS domain containing *L. major* PGK exhibits its optimum activity at pH 5.5. Thus, PAS domain

plays crucial role in pH dependent activity of this protein. To find out the molecular mechanism behind these phenomena, ATP and ADP dependent PGK activity between wild type and truncated enzymes were compared at pH7.5 (Fig. 26). At pH 7.5 wild type protein revealed saturation kinetics till 1 mM ATP concentration and very high ATP concentration inhibit PGK activity. Likewise, truncated enzyme at pH 7.5 showed saturation kinetics up to 1mM ATP concentration and displayed optimal activity at 1mM ATP concentration. Higher concentration of ATP acts as inhibitor against truncated enzyme at pH 7.5. All the kinetic parameter Vmax, Km, Ki are measured applying substrate inhibition model equation [Y = Vmax*X/(Km + X*(1 + X/Ki))] in PRISM 6 software. Measured kinetic parameter of both wild type and truncated enzyme at pH 7.5 are mentioned in Table 13.

Table 13: Kinetic activity Measurements at pH 7.5

Apparent rate constants from substrate and product inhibition model At pH 7.5				
enzyme	V_{max} (S^{-1})	k_m (mM)	k_i (mM)	ADP IC 50 (mM)
Full length	3.2± 1	0.54± 0.2	1.3± 0.7	0.5± 0.02
Truncated (Δ 115)	14± 6	2± 0.9	0.1± 0.04	0.9± 0.05

3.1.6. Higher concentration of ATP and ADP acts as inhibitor

From the calculated kinetic parameter it was found that the Ki value of LmPAS-PGK was 0.26 mM at pH 5.5 and the Ki value of truncated enzyme was 0.44 mM at pH 5.5. This result suggesting that in acidic pH Ki values for ATP in full length and truncated enzyme wasindistinguishable. Conversely, the Ki values were 0.1 mM for PAS domain deleted enzyme and 1.3 mM for LmPAS-PGK at pH 7.5. These results indicate that the Ki value in full-length enzyme for ATP was approximately 13 times greater than PAS domain deleted enzyme, which is significant (Fig.26). In product inhibition investigation, various concentrations of ADP (product) were used in presence of 1mM ATP in the assay mix at neutral pH and acidic pH. Both the enzyme LmPAS-PGK and PAS domain deleted enzyme was inhibited with increasing concentration of ADP at both pH 7.5 and pH 5.5. All the product inhibition model data were suited by utilizing a hyperbolic decay equation operating with ORIGIN 6.0 software and listed in Table14 and 15. IC50 (Half-maximal inhibitory concentration) value of wild type enzyme was 1.1 mM where IC50 value of truncated enzyme was 1.3 mM at acidic pH. This IC50 values are suggesting that ADP dependent inhibition in the two enzymes are similar at pH 5.5. At pH 7.5 the IC50 value of wild type enzyme for ADP was 0.5 mM which was closed to truncated enzyme, 0.9 mM. From the entire observation and measurements, indicating that the Ki (inhibition constant) value for substrate ATP is notably switched in full length enzyme in contrast to PAS domain deleted enzyme at neutral pH, on the other hand the IC50 for ADP (product)

is unchanged. Here, it can be concluded that higher concentration of ATP and ADP acts as inhibitor at both neutral pH 7.5 and acidic pH 5.5.

3.1.7. Discussion

This chapter demonstrates that higher concentration of substrate (ATP) and higher concentration of product (ADP) acts as inhibitor, where PAS domain plays a crucial role in PGK catalytic activity switching. On the basis of PGK catalytic activity aspects, it was found that (a) both the full length and truncated enzymes exhibited saturation kinetics up to 1mM ATP concentration but higher concentration of ATP inhibits the PGK activity at both pH 5.5 and pH 7.5. (b) In acidic pH the k_i value for ATP in wild type and truncated enzymes were similar, whereas at neutral pH the k_i value for ATP in the full length LmPAS-PGK was 13 times higher than the PAS domain deleted truncated enzyme. (c) In case of product inhibition, IC_{50} values in both enzymes displayed no significant difference at both neutral pH 7.5 and acidic pH 5.5.

Overall observation demonstrates that the k_i value for substrate ATP is notably changed in PAS domain containing protein in comparison to PAS domain deleted enzyme at neutral pH 7.5. So, PAS domain plays a crucial role in lower pH dependent PGK activity switching. Kinase activity was changed with substrate ATP at neutral pH 7.5. Cofactor Mg^{2+} ion are essential in kinase activity through substrate binding. Immediate question arises whether cofactor Mg^{2+} ions have any role in lower pH dependent PGK activity switching. In next chapter it will discuss about the role of Mg^{2+} ion and other divalent cations in LmPAS-PGK activity at various pH.

CHAPTER 2

" Role of cofactor Mg2+ ion and divalent cations inLmPAS-PGK "

RESULTS (Chapter 2)

"Role of cofactor Mg2+ ion and divalent cations in LmPAS-PGK"

3.2.1. Background

3.2.2. Mg^{2+} act as an inhibitor of full length LmPAS-PGK at neutral pH 7.5

3.2.3. Role of other divalent cation

3.2.4.Discussion

3.2.1. Background

To sense the surrounding environment, the PAS domain in various sensory enzymes is familiar to bind with different small molecules which includes different divalent cations [45, 186-188].Divalent cation has significant influence in different enzymatic activity, cellular functions, polymerisation,and promote agonist receptor interactions [188-190].PAS domain from different organisms, are capable of binding different divalent metal ions. Binding of divalent cations facilitates different protein functions and modulate numerous signalling pathway[191]. In transmemebrane of*E.coli*, PAS domain containing sensor kinase PhoQ and its associated partner PhoP acts as multiple function regulators that monitor different signalling events like Mg^{2+} transport, type III secretion, low pH sensing, acid stress resistance and cell wall structure remodelling [192, 193]. In low divalent metal ion concentrations[194], metal ions cannot bind to the transmembrane PAS domain of PhoQ sensor kinase and as a consequence, PhoQ activates PhoP. The pathogenic organisms adapt at different environment conditions including low divalent cation as well as acidic pH by using PhoQ-PhoPsignalling events [54]. In PhoQ sensor kinase, acidic cluster of residues in the PAS domain is essential for sensing the divalent metal ions and keep continuing the PhoQ sensor kinase in a repressed state [45].The PAS domain containing transmembrane histidine kinase sensor protein (PhoQ) has been described to interact with divalent metal ions via aspartic acid and glutamic acid residues present in the acidic patch of PAS domain[52, 195]. This interaction facilitates formation of bridge with inner membrane phospholipids to maintain the repression state of PhoQ. NMR study exhibit that PhoQ repression can be withdrawn by dislocate of divalent metal ions from acidic patch by acidic pH. In order to find out whether cofactor Mg^{2+} or other divalent cations binds at PAS domain of LmPAS-PGK and follow the same mechanism for repression at neutral pH 7.5. This chapter will discuss how Mg^{2+}ion and other divalent metal ions interact with PAS domain and inhibit LmPAS-PGK activity at neutral pH 7.5. To validate that full-length protein, PAS domain deleted protein, and yeast PGK (having lack of PAS domain) were utilized at different pH values.

3.2.2. Mg^{2+} act as an inhibitor of full length LmPAS-PGK at neutral pH 7.5

Our goal is to find out whether the inhibition of wild type PGK activity occurs directly when it interacts with divalent cation Mg^{2+} at pH 7.5. To securing this purpose. Comparative study of PGK activity among PAS domain containing PGK, PAS doman deleted PGK and yeast PGK (which is lacking of PAS domain) were done at various pH.

It was observed that the catalytic activity of PAS domain containing PGK was decreased with rising concentration of divalent cation Mg^{2+} ion at neutral pH 7.5 (Fig. 27) whereas the PGK catalytic activity of full length protein is increased with rising concentration of cofactor Mg^{2+} ion at pH 5.5. Conversely the rest two enzyme PAS domain deleted protein and yeast PGK exhibited that the activity of enzyme is directly proportional to Mg^{2+} ion

at both acidic pH 5.5 and neutral pH 7.5 (Fig. 27). Together, these data indicate that Mg^{2+} ions might be dissociated from PAS domain at the acidic pH 5.5 and subsequently, the Mg^{2+} induced autoinhibitory role of PAS domain is suppressed in the full length LmPAS-PGK at acidic pH 5.5. The concentration of Mg^{2+} cation did not change during catalysis, so results determined a K_D value (dissociation constant) instead of K_m from half saturation kinetics curve (Fig. 27 A-C). In case of full length LmPAS-PGK protein, the accurate K_D value of Mg^{2+} ions were unable to measure for catalytic site due to very low value (<0.01 mM) at pH 7.5, whereas, the K_D value (0.21 mM) of Mg^{2+} ion can be measured for regulatory site from inhibition kinetics at pH 7.5. The K_D values for truncated enzyme and yeast PGK at pH 7.5 were 0.10 mM and 0.13 mM, respectively. These data indicated that the dissociation constant for Mg^{2+} binding in the catalytic site of full length enzyme was lower compared to both truncated enzymes and yeast PGK at neutral pH. On the other hand, the K_D values for full length, truncated PAS-PGK, and yeast PGK at pH 5.5 were 0.6 mM, 0.17 mM and 0.7 mM, respectively, suggesting that the dissociation constant for Mg^{2+} cation in the catalytic site ofall PGKs was similar order at acidic pH.

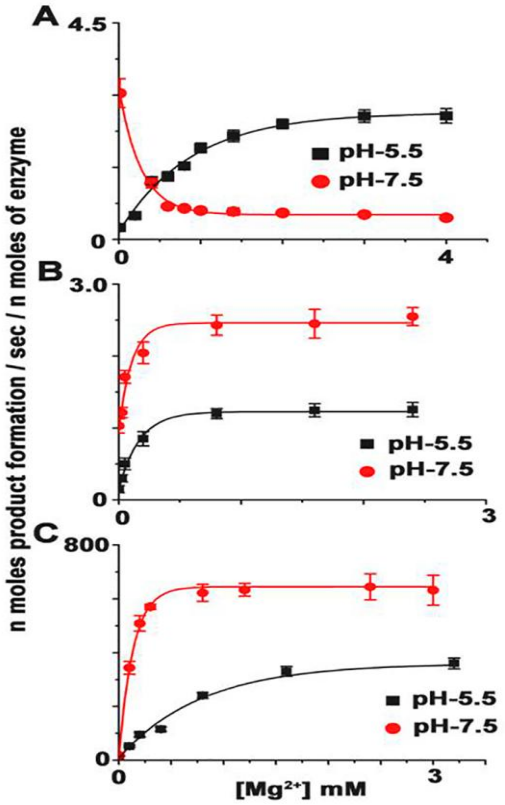

Fig. 27. Comparative studies of divalent cation-dependent PGK activity among full length proteins (Panel A), PAS deleted proteins (Panel B), and yeast PGK (lacking PAS domain; Panel C) at various pH values (7.5 and 5.5).

3.2.3 Role of other divalent cation

To find-out the role of other divalent cation in full length LmPAS-PGK activity, the phosphoglycerate kinase activity was measured in presence of various conventional divalent cations like Ca^{2+} and Mn^{2+} along with Mg^{2+}.

At neutral pH 7.5, quite low concentration of Mg^{2+} (0.01 mM) shows the highest activity, while thehigher concentration of Mg^{2+} ion (2 mM)inhibits PGK activity. Higher concentration of divalent cation Ca^{2+} (2mM) exhibit the highest PGK activity at neutral pH 7.5. In contrast to Ca^{2+} ion, the effect of Mn^{2+} ion is similar to Mg^{2+} ions (Fig. 28). While PAS domain deleted protein exhibit that the highest concentration of Mg^{2+} (2mM) and Mn^{2+}(2mM) ionsare not capable of inhibiting the PGK catalytic activity at neutral pH

7.5. In yeast PGK (lack of PAS domain), it was found that low concentration of Mg^{2+} exhibit low PGK catalytic activity whereas with increasing concentration of Mg^{2+} ion its catalytic activity increased. The same pattern of results also found when treated with Mn^{2+}.

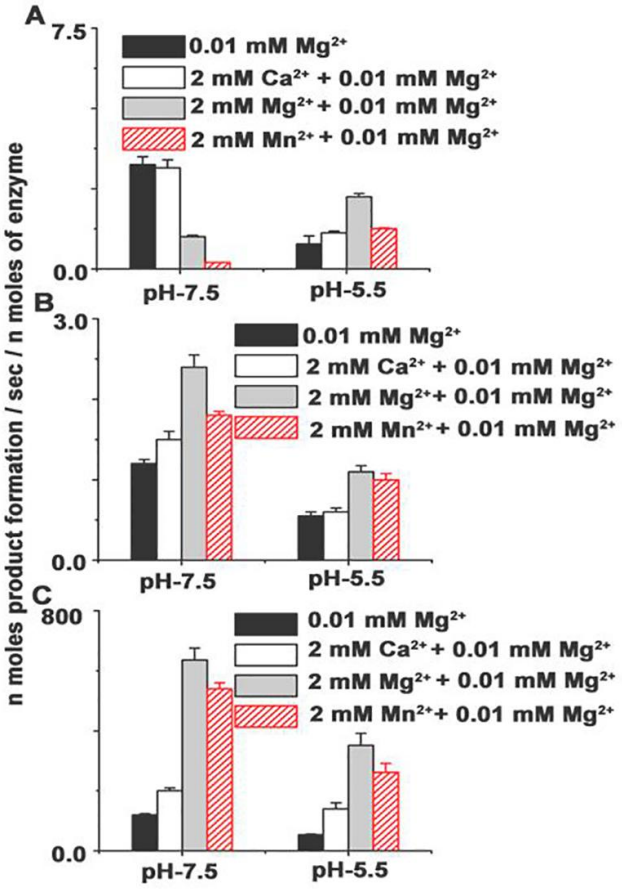

Fig.28. Comparative studies of divalent cation-dependent PGK activity among full-length proteins (Panels A), PAS deleted proteins (Panels B), and yeast PGK (lacking PAS domain; Panels C) at various pH values (7.5 and 5.5).

The overall results indicate that the activity of wildtype full length protein was not sensitive to Ca^{2+} ion because the divalent cation Ca^{2+} may not be able to bind with PAS domain. Conversely, Mn^{2+} act just like Mg^{2+} ion and which can able to bind with PAS domain and facilitates the inhibition of the PGK catalytic activity at neutral pH 7.5.

3.2.4. Discussion

This chapter discussed the role of divalent cation Mg^{2+} ion and other divalent cations in PGK catalytic activity at different pH values. On the basis of PGK catalytic activity with different divalent cations at different pH it was found that, (a) the catalytic activity of LmPAS-PGK protein is decreased with increasing concentration of cofactor Mg^{2+} or Mn^{2+} at the neutral pH 7.5, (b) inhibition by higher concentration of Mg^{2+} or Mn^{2+} (2mM) at neutral pH is insensitive to both PAS domain deleted protein and yeast PGK (lacking PAS domain), (c) The K_D value (dissociation constant) for Mg^{2+} ion binding in the catalytic site of wild type LmPAS-PGK was lower in comparison to both PAS domain deleted PGK and yeast PGK at pH 7.5. (d) The K_D values for Mg^{2+} in full length LmPAS-PGK, truncated enzyme and yeast PGK were similar at acidic pH 5.5.

The above observation indicates that the divalent cation Mg^{2+} ion might be released form N-terminal PAS domain at acidic pH 5.5, as a consequence the autoinhibitory role of PAS domain is repressed in full length LmPAS-PGK at pH 5.5. The activity of wild type protein was not sensitive to Ca^{2+} ion because PAS domain may not able to bind with Ca^{2+}

ion. Both $Mg^{2+}$and Mn^{2+} ionswere able to bind with PAS domain and inhibited the PGK catalytic activity at neutral pH 7.5.

The obvious question immediately arises where the Mg^{2+} ion binds in PAS domain. In our next chapter it will discuss about key residue where the Mg^{2+} ion binds and how this residue in PAS domain play crucial role for the repression of PAS domain mediated catalytic PGK domain.

CHAPTER 3

"Role of Histidine 57 in the repression of PAS domain mediated catalytic PGK domain

RESULTS (Chapter 3)

“

“Role of Histidine 57 in the repression of PAS domain mediated catalytic PGK domain”

3.3.1. Background

3.3.2 Cloning, expression and Purification of Histidine mutatnt proteins

3.3.3. Comparative study of His mutant proteins substrate binding affinity and catalytic activity with wild type enzyme

3.3.3.1.i) Substrate binding affinity

3.3.3.2.ii) Catalytic activity

3.3.4. Comparative study of His mutant proteins in presence of different divalent cations at pH 5.5 and pH 7.5

3.3.5.Validation of LmPAS-PGK and Mg^{2+} interactions by Fluorescence spectroscopy and Isothermal titration calorimetry

3.3.5.1.i) Tryptophan Fluorescence Spectroscopy

3.3.5.2.ii) Isothermal titration calorimetry

3.3.6.Discussion

3.3.1 Background

Earlier work indicated that the optimum activity of LmPAS-PGK was near pH 5.5 [182], where other PGK show their optimum activity near pH 7.5. Among all the amino acids only histidine has pKa value near pH 6.0 [196].Histidine residues get protonated below pH 6.0. Thus, on the basis of these datait washypothesized that the protonation-deprotonation of a histidine residue in a critical region of the PAS domain might act as the conformational switch associated with the activation. Four histidine residues are present in the primary structure of the PAS domain of the wild type protein. The position of the histidine residues present in PAS domain is 57, 71, 89 and 111. To examine the possibility that protonation of one or more of the histidine residues is responsible for pH-mediated catalytic PGK domain activation, all four His residues are changed to Ala and tested all four mutant proteins for divalent metal ion-dependent PGK activity at various pH.

3.3.2. Cloning, expression and purification of histidine mutant proteins

In order to addressing the biochemical and biophysical characteristics of all four histidine mutants, the four His to Ala single mutants were expressed in *E. coli*. Recombinant H57A, H71A, H89A and H111A mutants carrying an N-terminal six-histidine tag were purified using Ni^{2+}-NTA column. Purified H57A, H71A, H89A and H111A mutant proteins were migrated to 60 kDa position in SDS-PAGE, as same as the molecular mass of wild type full length enzyme (Fig.29).

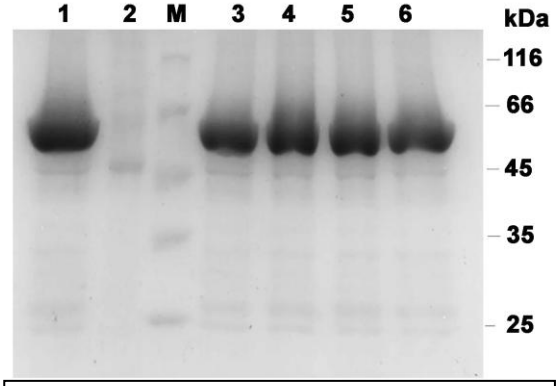

Fig.29. Purification of recombinant *L. major* full length PAS-PGK, Δ115LmPAS-PGK and H57A, H71A, H89A, H111A mutant proteins shown in a Coomassie Blue stained 13% SDS-PAGE analysis of protein fractions SDS-PAGE; Lane 1: full length PAS-PGK,Lane 2: Δ115LmPAS-PGK, Lane 3:Molecular weight Marker, Lane 4: H57A, Lane 5:H71A, Lane 6: H89A, Lane 7: H111A

3.3.3. Comparative study of His mutant proteins substrate binding affinity and catalytic activity with wild type enzyme

3.3.3.1. i) Substrate binding affinity:

Four single histidine mutant proteins were created and measure the substrate binding affinity and catalytic activity of all four H57A, H71A, H89A and H111A mutant proteins. Comparative study of these mutant proteins was done with wild type proteins. Figure 30 exhibits that wild type protein along with all histidine mutant proteins follows standard Michaelis–Menten kinetics with respect to both of its substrate ATP and 3-PGA. In Table 14 $k_{cat}$and Km values of all histidine mutants and wild type protein were compared at both pH pH 7.5 (Table 14) and pH 5.5 (Table 15).

Table14: Catalytic activities of wild type and mutants LmPAS-PGK proteins at pH 7.5.

Enzyme	Catalytic activity parameters			
	K_m (μM)		k_{cat} (s^{-1})	
	ATP	3PGA	ATP	3PGA
LmPAS-PGK	152 ± 4	530 ± 25	1.4 ± 0.10	1.37 ± 0.12
H57A LmPAS-PGK	156 ± 8	511 ± 34	4.5 ± 0.2	4.4 ± 0.3
H71A LmPAS-PGK	122 ± 5	490 ± 41	1.28 ± 0.05	1.3 ± 0.0.05
H89A LmPAS-PGK	139± 10	517 ± 45	1.35 ± 0.09	1.35 ± 0.08
H111A LmPAS-PGK	163 ± 14	480± 29	1.3 ± 0.10	1.29 ± 0.1

It was found that the Km values of the wild-type protein,H57A, H71A, 89A and H111A for ATP at pH 7.5 were 152 μM, 156 μM, 122 μM, 139 μM and 163 μM, respectively. These data suggest that the Km value of wild type protein was very similar to all histidine mutant proteins. The Km values of the wild-type protein, H57A, H71A, 89A and H111A for 3PGA at pH 7.5 were found 530 μM, 511 μM, 490 μM, 517 μM and 480 μM, respectively. So, at pH 7.5 the Km values of wild type protein and all His to Ala mutant proteins have almost similar.

Table15. Catalytic activities of wild type and mutants LmPAS-PGK proteins at pH 5.5.

Enzyme	Catalytic activity parameters			
	K_m (μM)		k_{cat} (s^{-1})	
	ATP	3PGA	ATP	3PGA
LmPAS-PGK	118 ± 4	661 ± 19	3.5 ± 0.13	3.7 ± 0.2
H57A LmPAS-PGK	134 ± 8	610 ± 24	3.8 ± 0.2	3.6 ± 0.16
H71A LmPAS-PGK	102 ± 5	680 ± 50	3.3 ± 0.3	3.2 ± 0.1
H89A LmPAS-PGK	125 ± 4	630 ± 38	3.2± 0.15	3.2 ± 0.3
H111A LmPAS-PGK	128 ± 9	638 ± 29	3.6 ± 0.23	3.8 ± 0.18

The Km values of the wild-type protein,H57A, H71A, H89A and H111A for ATP at pH 5.5 were 118 μM, 134 μM, 102 μM, 125 μM and 128 μM, respectively (Table15). These data suggest that the Km value of wild type protein for ATP at pH 5.5 did not alter after histidine mutation at position 57, 71, 89 and 111. The Km values of the wild-type protein, H57A, H71A, H89A and H111A for 3-PGA at pH 5.5 were 661 μM, 610 μM, 680 μM, 630 μM and 638 μM, respectively (Table15). Alltogether, these results demonstrated that alteration of histidine residues by alanine in four different position did not exhibit any effect on the Km values of both the substrates ATP and 3-PGA at both H 5.5 and 7.5.

3.3.3.2. ii) Catalytic activity

The enzymatic activity (k_{cat}) of the wild-type protein, H57A, H71A, H89A and H111A at acidic pH 5.5 were 3.5 s^{-1}, 3.8 s^{-1}, 3.3 s^{-1}, 3.2 s^{-1} and 3.6 s^{-1}, respectively (Table 15 and Fig. 30).These data suggest that all the His mutant proteins also show similar catalytic

activity compared to wild type protein. While, the enzymatic activity of wild type, H57A, H71A, H89A and H111A protein at neutral pH 7.5 were 1.4 s^{-1}, 4.5 s^{-1}, 1.28 s^{-1}, 1.35 s^{-1} and 1.3 s^{-1}, respectively (Table 14 and Fig. 30). These data suggest that H57A showthree-fold greater enzymatic activity at neutral pH 7.5 compared to wild type protein.

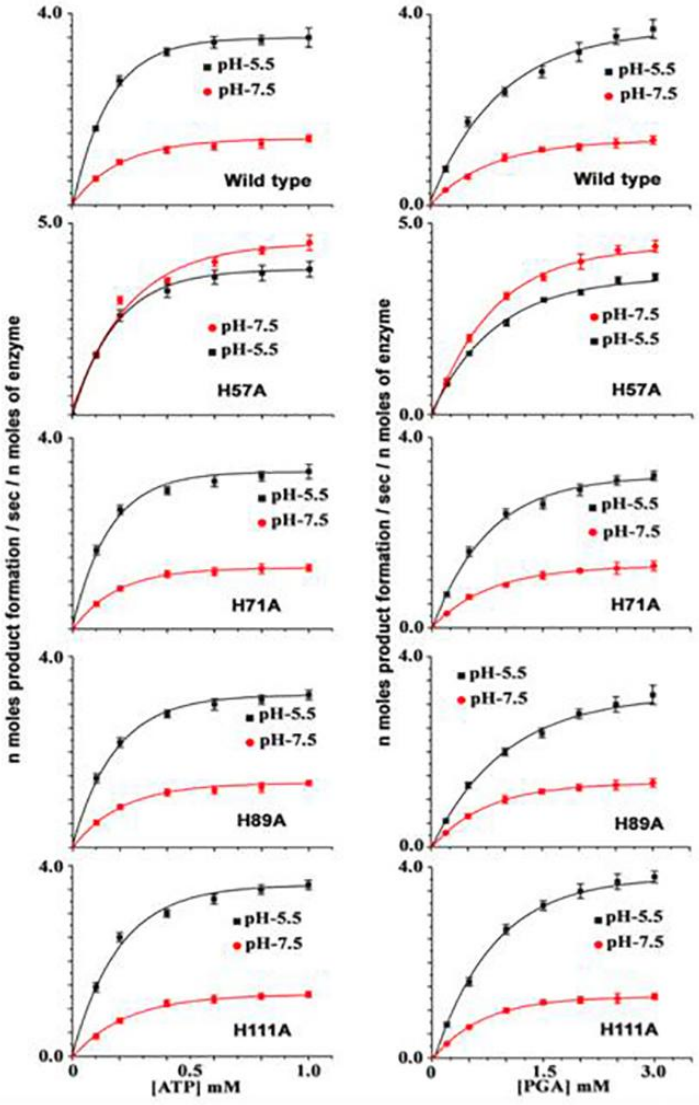

Fig. 30. Velocity of wild-type, H57A, H71A, H89A, and H111A proteins with varying concentrations of ATP or 3-PGA. All data are fitted to a hyperbolic curve. The K_m and k_{cat} values from the fitting curve are listed in Table 16 and 17. Data were plotted as means ± SD from three independent experiments for each assessment.

All these results demonstrated that H57A residue of the PAS domain is not directly involved in substrate binding, while this residue is responsible for the repression of the PGK catalytic activity at neutral pH 7.5.

3.3.4.Comparative study of His mutant proteins in presence of different divalent cations at pH 5.5 and pH 7.5

Enzymatic activity of all His mutant proteins were measured in presence of different divalent cations at pH 5.5 and pH 7.5. It was found that PGK activity of H71A, H89A, and H111A mutant proteins show similar activity compared to wild type protein.

Unlike wild type LmPAS-PGK protein, the catalytic activity of the H57A out of four His mutants were directly proportional to the concentration of Mg^{2+} ions at neutral pH 7.5 (Fig. 31). Thus, the H57A mutants are derepressed at pH 7.5 compared to wild-type as well as other His mutants (H71A, H89A and H111A) in the presence of either 2.0 mM

Mg^{2+} ionsor 2.0 mM Mn^{2+} ions. The loss of the imidazole side chain at position His57 by

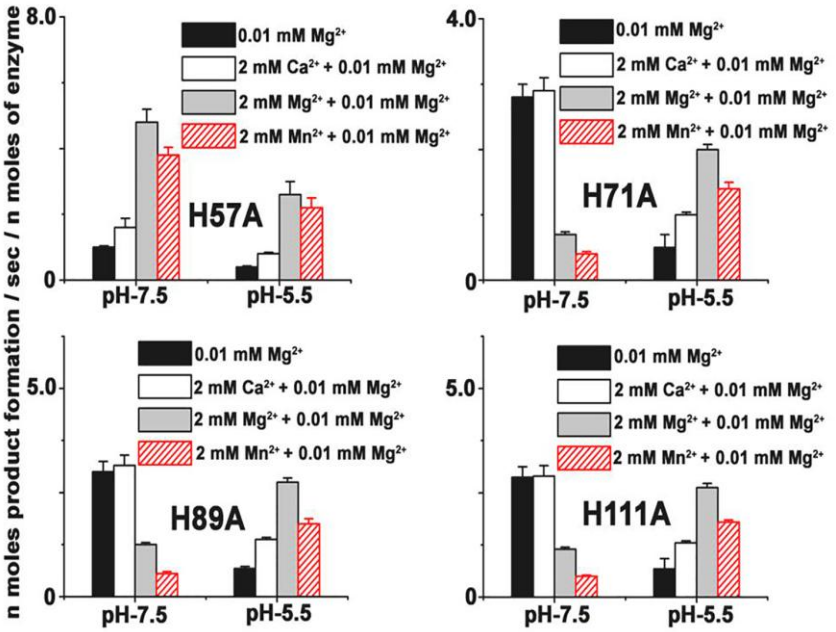

Fig.31. Comparative studies of divalent cation-dependent PGK activity among H57A, H71A, H89A, and H111A at various pH values (7.5 and 5.5).

Ala destabilizes specifically the repressed state. Thus, the presence of a histidine at position 57 is mandatory to keep wild-type repression of LmPAS-PGK through cofactor Mg^{2+} binding in PAS domain. Taken together these results indicate that activation by acidic pH is a result of protonation of His 57 not His71, His89 and His111 residue.

3.3.5. Validation of LmPAS-PGK and Mg^{2+} interactions by Fluorescence spectroscopy and Isothermal titration calorimetry:

3.3.5.1. i) Tryptophan Fluorescence Spectroscopy

The primary sequence of LmPAS-PGK contains three tryptophan residues; one in the PGK catalytic domain (Trp445), and two in the PAS domain (Trp40 and Trp95). The optimum tryptophan fluorescence of LmPAS-PGK occurred at 331 nm (Figure 32A). As binding of Mg^{2+} ion is a prerequisite for repression, the interaction of Mg^{2+} ion with wild type as well as H57A LmPAS-PGK were analyzed by tryptophan fluorescence spectroscopy. Titration of enzyme with Mg^{2+} ion at pH 7.5 (Figure 32A), resulted in quenching of the tryptophan fluorescence emission maxima in a divalent Mg^{2+} ions concentration-dependent manner (Figure 32A). Such quenching of tryptophan fluorescence emission maxima, known to result from the tryptophan becoming more opened up from the hydrophobic atmosphere, is indication of changes to the micro-environment around the tryptophan residue. Once the changes of buffer control titrations ran in parallel were deducted, the maximum changes of the intrinsic fluorescence intensity by ligand binding were 1500 units for Mg^{2+} ions. The difference of the tryptophan fluorescence in response to titration of the enzyme-Mg^{2+} complexes vs Mg^{2+} concentration was plotted (Figure 32A, inset) and the apparent equilibrium dissociation

constant (K_D) for Mg^{2+}ions were 0.3 mM ± 0.01 mM. The tryptophan fluorescence intensity of the H57A changed by the addition of Mg^{2+} ions was a very small reduction of fluorescence intensity due to buffer dilution (Figure 32B). This provides further support for the involvement of His57 residue in divalent Mg^{2+} ion binding in the PAS domain of LmPAS-PGK.

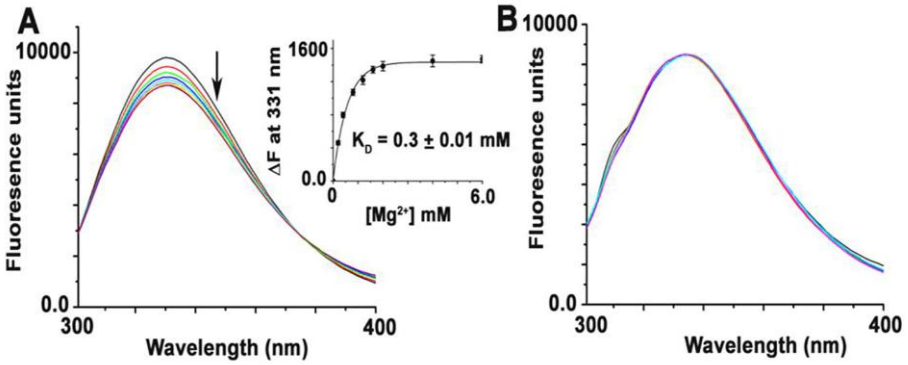

Fig. 32.Determination of wild-type and H57A LmPAS-PGK-Mg2$^+$ interactions by tryptophan fluorescence spectroscopy and ITC at pH 7.5

3.3.5.2. ii) Isothermal titration calorimetry

To compare the divalent cation binding characteristics obtained by tryptophan fluorescence spectroscopy, the interactions of enzyme with Mg^{2+} ions were also measured by isothermal titration calorimetry (ITC) at pH 7.5. The ITC titration traces were fitted well into a single-site binding model for Mg^{2+} ion binding (Fig. 33A) and the apparent equilibrium dissociation constant (K_D) for Mg^{2+}ions were 0.5 mM ± 0.05 mM (Fig. 33A). Mg^{2+} ions binding to His57Ala mutant were too weak to be reliably determined by ITC (Fig. 33B). The K_D values of the binding of Mg^{2+} ion to wild type full length protein revealed

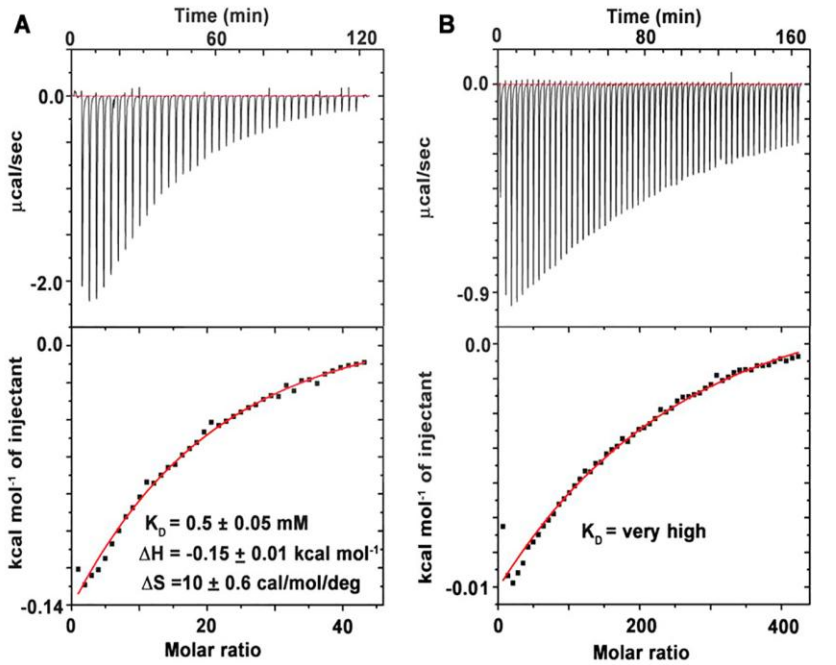

Fig. 33. Determination of wild-type and H57A LmPAS-PGK-Mg^{2+} interactions by Isothermal titration calorimetry at pH 7.5 Heat changes for titration of wild-type (Panel A) and H57A-LmPAS-PGK (Panel B).

here by ITC are in well-fitted with the dissociation constants (K_D) obtained by tryptophan fluorescence spectroscopy. Further analyses of the thermodynamics of wild type protein and the Mg^{2+} ion interaction demostrate that Mg^{2+} ion binding to wild type protein is enthalpically-driven, with a high negative enthalpy, ΔH, of -0.15 kcal/mol (Fig. 33A), whereas, the Mg^{2+} ion binding to H57A protein is less favourable binding enthalpy (ΔH), of -0.02 kcal/mol (Fig. 33B).

3.3.6. Discussion

Here, some salient points of this chapter are: (a) His 57 in PAS domain plays crucial role for the Mg^{2+} or Mn^{2+} dependent repression of catalytic PGK domain, (b) tryptophan fluorescence spectroscopy analysis and isothermal titration calorimetry experiments indicate that His 57 in PAS domain is crucial for the Mg^{2+} ion binding. Taken together, a central finding is reaching from above salient points is that the network of interaction that includes H57 is indispensable for divalent cation-mediated repression and is also associated with pH-mediated activation. This result is consistent with other groups of work because similar type of divalent cation binding has been previously observed in PAS domains of other proteins, such as PhoQ from *Salmonella typhimurium* and WalK from *Streptococcus aureus*, which senses the cations Ca^{2+}, Mg^{2+} and Zn^{2+} etc [98, 197].

Conclusion & Perspectives

CONCLUSION & PERSPECTIVE

Generally most of all PGKs exhibit its optimal catalytic activity at near about pH 7.5. Yet, wild type LmPAS-PGK shows its optimal catalytic activity at pH 5.5. This feature makes it unique, this thesis is trying to understand the molecular mechanism behind unique phenomenon and how PAS domain mechanistically help for this distinct feature of full length LmPAS-PGK. The entire observations suggest that (a) the LmPAS-PGK can directly activated by acidic pH (pH 5.5), (b) pH change have no role on the response of LmPAS-PGK to product (ADP) inhibition, (c) the PGK catalytic activity of full length LmPAS-PGK is reduced with increasing concentration of divalent cation Mg^{2+} or Mn^{2+} at neutral pH 7.5, (d) higher concentration of cofactor Mg^{2+} or Mn^{2+} (2 mM) is insensitive to inhibit the catalytic activity of the PAS domain-deleted protein as well as yeast PGK at neutral pH 7.5, (e) His 57is the key residue in the PAS domain which plays pivotal role for the Mg^{2+} or Mn^{2+} dependent repression of C-terminal catalytic PGK domain, (f) tryptophan fluorescence spectroscopy and ITC experiments further confirm that His 57 is the key residue in the PAS domain which is crucial for the Mg2+ ion binding. The central finding from the above salient points is that H57 is vital for divalent cation mediated repression and pH-mediated activation. This type of divalent cation binding has been distinguished earlier in PAS domains of other proteins, like PhoQ from *Salmonella typhimurium* and WalK from *Streptococcus aureus*, which senses the cations Ca^{2+}, Mg^{2+}, Zn^{2+}, etc. [98, 197].

Mg^{2+} ion acts as an essential cofactor for the kinase activity. Even though very little concentration of Mg^{2+} ions exhibits it optimum catalytic activity at pH 7.5. In presence of metal ion chelator EDTA, full length, LmPAS-PGK does not exhibit any catalytic activity of PGK which specifying that Mg^{2+} ion is crucial cofactor for the PGK activity. The ^{31}P NMR analysis of a RhATP-PGA-PGK complex [198] and the closed form of PGK crystal structure [199] proposed that in the presence of the substrate 1,3-bisphosphoglycerate, the cofactor Mg^{2+} ion switches from coordinating the α- and β-phosphates of ADP to bridging the β-phosphate of ADP and the 1-phosphate of 1,3-bisphosphoglycerate. Inclusively full length LmPAS-PGK has two binding sites for cofactor Mg^{2+} ions, first in the active site of PGK domain (through ADP binding) and another one is in the PAS domain (through His57 residue). One of the possible roles of H57 in divalent cation-mediated repression as well as acidic pH-mediated activation is that either H57 is required for divalent cation binding, which forms bridge between regulatory PAS domain and catalytic PGK domain, or the H57 indirectly controls divalent cation binding, which regulates pH-dependent PGK activation.

The immediate question comes what is physiological significance of the uniqueness, observed inhibition are seemingly involved in the *Leishmania* survival. Gene knockout

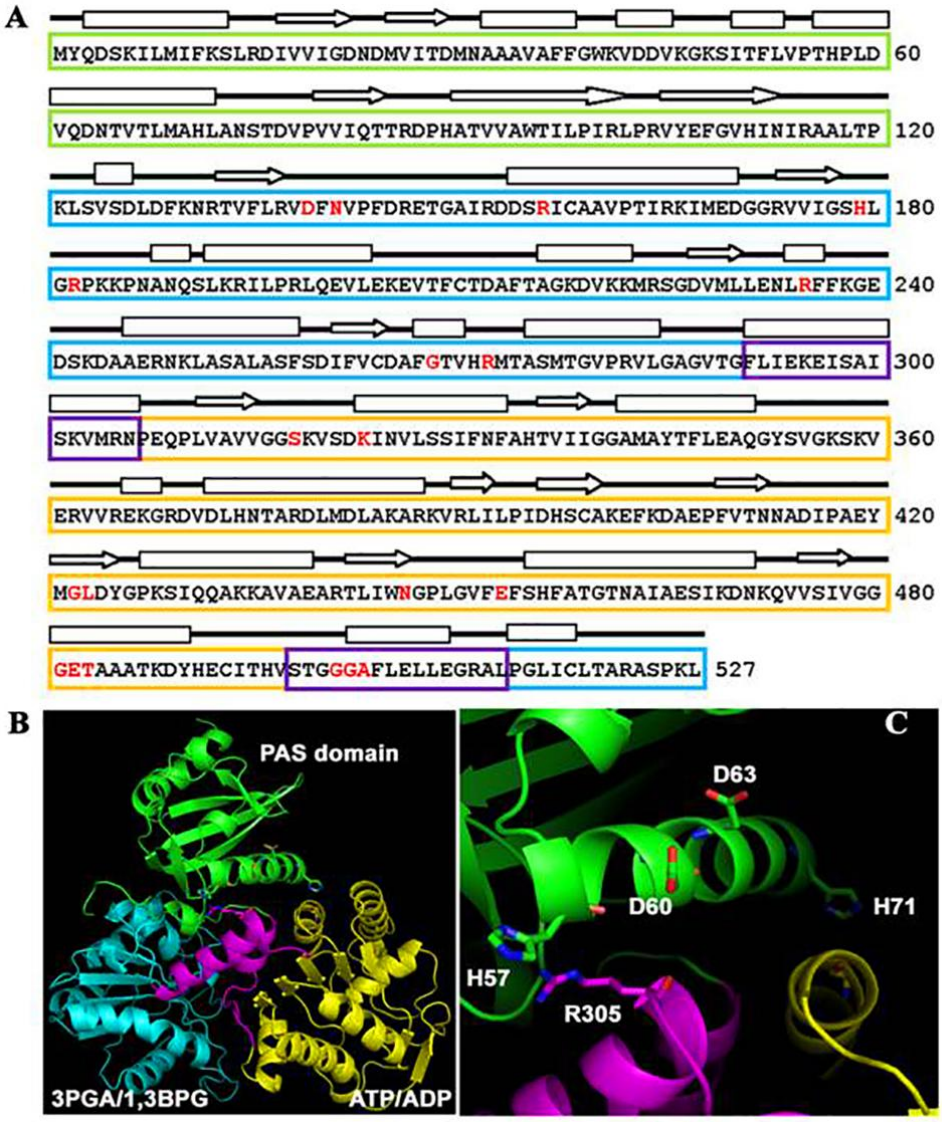

Fig.34. Model illustrating the potential interaction site between regulatory PAS domain and catalytic PGK domain. Secondary structure with active-site residues (red color) (A) and ribbon representation (B) of LmPAS-PGK model structure determined from the X-ray structure of closed form of *Trypanosoma brucei* phosphoglycerate kinase (PDB ID: 13PK) as well as the crystal structure of PAS domain of *Streptococcus aureus* WalK protein (PDB ID: 4MN5). Above, black arrows show β strands, and empty boxes show α-helices. The PAS domain is green (residues 1–120), the N-terminal of PGK domain (residues 121–290 and 513–527) is cyno, the Cterminal of PGK domain (residues 307–497) is yellow, and the hinge region (residues 291–306 and 497–512) is purple. The hinge regions are critical for conformational change and catalysis. This model structure was done by knowledge-based homology modeling using the Modeller and PyMOL software. (C) is the same as (B) after magnification.)

studies suggest that LmPAS-PGK has a protecting role at longer durations of culture media where unfavorable growth conditions are occurred like nutrient stress, acidic pH, or toxic metabolite (182). Normally, the concentration of cytosolic Mg^{2+} ion is ~ 1 mM, which is enough for optimum ATP generation by the standard kinase enzyme in the parasite, but Mg^{2+} or Mn^{2+} deficiency leads to the lowering of ATP generation for

slowing down the cell growth. The Mg^{2+}- or Mn^{2+}-sensitive LmPAS-PGK may supply ATP under metal ion stress and thus can help in the survivability of these intracellular pathogens.

Till now, there is no available X-ray crystal structure of PAS domain-containing PGK protein from any other organisms in the literature. The modeller software predicts a modelled structure of full length LmPAS-PGK (Fig. 34). His57 residue is present on the α5 helix of the N-terminal PAS domain of full length LmPAS-PGK, which locates near hinge region of the catalytic PGK domain. Asp60 and Asp63 present in the acidic patch of the α5 helix, this is the possible binding site of divalent cation in PAS domain containing LmPAS-PGK. The Mg^{2+} ions are hypothesized to form connected bridges between the regulatory PAS domain and the catalytic PGK domain.

The experimental data exhibit that PGK domain was activated at neutral pH by displacement of cofactor Mg^{2+} ion from the PAS domain in H57A mutant. This type of structural change is able to destabilize the Mg^{2+} bridge which maintain the repression of the PGK catalytic activity. The H57 mediated interaction network might be need for maintaining divalent cation mediated repression, presumably via structural rigidity that holds the hinge region of the PGK domain, because despite the Mg^{2+} cation bridge, the substrate-induced domain closer to PGK domain might be freely carried out during catalysis. To elucidate the complete network of interaction of divalent metal ion binding site through the X-ray crystal structure of divalent cation-bound PAS-PGK enzyme will be done in near future.

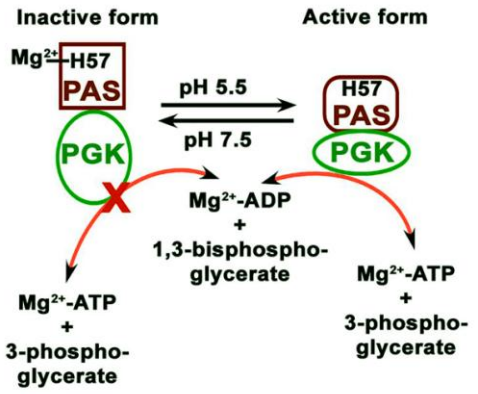

Fig: 35 Graphical representation of PGK activity switching through Mg^{2+} binding in H57 residue of PAS domain at acidic pH 5.5 and neutral pH 7.5

A schematic diagram shows the summarisation of themolecular mechanism of the H57 mediated regulation of catalytic activity in Leishmanial PAS domain containing phosphoglycerate kinase (Fig. 35). Active form to inactive form of this enzyme are switching during either alteration of pH or changing the divalent cation concentration; inactive form is Mg^{2+} bound at H57 in PAS domain of full length enzyme at neutral pH 7.5 and active form is Mg^{2+} free PAS domain of this enzyme at lower pH 5.5.

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