Characterization of Heat Shock Protein 70-z (PfHsp70-z) from *Plasmodium falciparum*

by

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ABSTRACT
Malaria is a parasitic disease that accounts for more than 660 thousand deaths annually, mainly in children. Malaria is caused by five Plasmodium species *P. ovale, P. vivax, P. malariae, P. falciparum* and *P. knowlesi*. The most lethal cause of cerebral malaria is *P. falciparum*. The parasites have been shown to up-regulate some of their heat shock proteins (Hsp) in response to stress. Heat shock protein 70 (called DnaK in prokaryotes) is one of the most prominent groups of chaperones whose role is central to protein homeostasis and determines the fate of proteins. Six *Hsp70* genes are represented on the genome of *P. falciparum*. The *Hsp70* genes encode for proteins that are localised in different sub-cellular compartments. Of these two occur in the cytosol, PfHsp70-z and PfHsp70-1; two occur in the endoplasmic reticulum, PfHsp70-2 and PfHsp70-y; one in the mitochondria, PfHsp70-3 and one exported to the red blood cell cytosol, PfHsp70-x. PfHsp70-1 is a well characterized canonical Hsp70 involved in prevention of protein aggregation and facilitates protein folding. Little is known about PfHsp70-z. PfHsp70-z was previously shown to be an essential protein implicated in the folding of proteins possessing asparagine rich repeats. However, based on structural evidence PfHsp70-z belongs to the Hsp110 family of proteins and is thought to serve as a nucleotide exchange factor (NEF) of PfHsp70-1. The main aim of this study is to elucidate the functional roles of PfHsp70-z as a chaperone and its interaction with PfHsp70-1. In the current study, PfHsp70-z was cloned and expressed in *E. coli* JM109 cells. This was followed by its purification using nickel chromatography. The expression of PfHsp70-z in parasites cultured *in vitro* was investigated and its association with PfHsp70-1 was explored using a co-immunoprecipitation assay. PfHsp70-z expression in malaria parasites is up regulated by heat stress and the protein is heat stable based on investigations conducted using Circular Dichroism. Furthermore, the direct interaction between recombinant forms of PfHsp70-z and PfHsp70-1 were investigated using slot blot and surface plasmon resonance assays. PfHsp70-z was observed to exhibit ATPase activity. In addition, the direct interaction between PfHsp70-z and PfHsp70-1 is promoted by ATP. Based on limited proteolysis and tryptophan fluorescence analyses, PfHsp70-z binds ATP to assume a unique structural conformation compared to the conformation of the protein bound to ADP or in nucleotide-free state. PfHsp70-z was able to suppress the heat-induced aggregation of malate dehydrogenase and luciferase *in vitro*. Interestingly, while ATP appears to modulate the conformation of PfHsp70-z, the chaperone function of PfHsp70-z was not influenced by ATP. Altogether, these findings suggest that
PfHsp70-z serves as an effective peptide substrate holding chaperone. In addition, PfHsp70-z may also serve as the sole nucleotide exchange factor of PfHsp70-1. The broad spectrum of functions of this protein, could explain this PfHsp70-z is an essential protein in malaria parasite survival. This is the first study to show that PfHsp70-z possess independent chaperone activity and that it interacts with its cytosolic counterpart, PfHsp70-1 in a nucleotide dependent fashion. Furthermore, the study shows that PfHsp70-z is a heat stable molecule and that it is capable of forming high order oligomers.

**Key words:** Malaria, *Plasmodium falciparum*, chaperone, nucleotide exchange factor, PfHsp70-z
DECLARATION

I Tawanda Zininga hereby declare that the thesis for the Doctor of Philosophy degree at the University of Venda, hereby submitted by me, has not previously been submitted for a degree at this or any other university, and that it is my own work in design and execution and that all reference material contained therein has been duly acknowledged.

Signature.................................................................Date.................................................................
DEDICATION

This thesis is lovingly dedicated to

My Wife Chipo, and our daughters Nadine and Eleora
PREFACE

This thesis is presented in form of six chapters whose outlines is provided below based on the various thematic areas covered in the study. Some aspects of the thesis have already been published in peer reviewed articles (see list of outputs; page xvii). The outline of the thesis chapters is as follows;

- **Chapter 1**: This is a general introduction encompassing all the background to the suggested broad aim and objectives. The broad problem statement, hypothesis, aim and objectives are highlighted in section 1.5.

- **Chapter 2**: This chapter presents the bioinformatics analyses of the structural features of PfHsp70-z.

- **Chapter 3**: This chapter presents the production of recombinant PfHsp70-z protein and biochemical analysis of its secondary and tertiary features.

- **Chapter 4**: This chapter presents investigation of the association of PfHsp70-z with PfHsp70-1 and assessment of its chaperone activity in vitro.

- **Chapter 5**: This chapter represents analysis of the expression, co-localisation and interaction of PfHsp70-z with PfHsp70-1 in parasites maintained at the red blood stage.

- **Chapter 6**: This chapter covers conclusive remarks and future perspectives.
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LIST OF SYMBOLS

Abbreviations of units Symbol Interpretation

% percent
μl microlitre
A320 absorbance at 320 nanometres
A360 absorbance at 360 nanometres
A595 absorbance at 595 nanometres
A600 absorbance at 600 nanometres
bp base pair
kDa kilodalton
μM micromolar
pM picomolar
°C degree Celsius
μl microlitre
ml millilitre
l litres
w/v weight per volume
v/v volume per volume
μg microgram
g gram
α alpha
β beta
LIST OF OUTPUTS

JOURNAL ARTICLES:


CONFERENCE PROCEEDINGS


5. **T. Zininga,** E. Prinsloo and A. Shonhai. Cytosolic *Plasmodium falciparum* heat shock protein 70-z (PfHsp70-z) interacts with PfHsp70-1 in a nucleotide dependent manner. University of Venda, Research Open day. 3-7 March 2015.
6. **T. Zininga**, and A. Shonhai. Analysis of the functional interaction between cytosolic *Plasmodium falciparum* heat shock protein 70-z (PfHsp70-z) and PfHsp70-1. The European Molecular Biology Organization (EMBO) Practical Course on Computational Analysis of Protein-Protein Interactions: from Sequences to Networks, September 29th to October 3rd 2014 at the University of Cape Town.
CHAPTER 1

INTRODUCTION

Literature Review
1.1 Human Malaria

1.1.1 Epidemiology

Malaria is contracted by more than 198 million people each year and more than 584 thousand people die yearly with the majority being in the tropics (WHO, 2014). Malaria is the most severe public health problem worldwide and largely affects sub-Saharan Africa. Africa accounts for about 90% of all deaths due to malaria, and of these 78% are children under 5 years (Orem et al., 2012; WHO, 2014). More than half the world population (3.4 billion people) lives in areas with high risk of malaria transmission spread over 106 countries and regions (CDC, 2014; WHO, 2014). Malaria causes economic and social burdens with a direct estimated cost (illness, treatment and premature death) of US$ 12 billion per year (Sachs and Malaney, 2002; CDC, 2014). The burden on individual countries starts as loss of income which translates to reduced economic growth resulting in reduced economic development of ~1% per year (CDC, 2014).

Human malaria is caused by *Plasmodium* species which are classified under the phylum Apicomplexan (Figure 1.1; Morrison, 2009). *Plasmodium* species are transmitted by female *Anopheles* mosquitoes. The initial documented description of malaria was made around 2700 BC in ancient Chinese documents. Subsequent studies proving that malaria was caused by protozoa were reported by Laveran in 1880 (Reviewed in Cox, 2010). Malaria was then described on the basis of periodicity (frequency of occurrence) and was thought to be caused by four species responsible for: benign tertian (*Haemamoeba vivax*), malignant tertian (*Laverania malariae*), quartan (*Heamamaeba malariae*) malaria and *P. ovale*. These species are now known as *P. vivax*, *P. falciparum* and *P. malariae*, respectively (Stephens, 1922 in Cox, 2010). *P. knowlesi* was initially considered a parasite for primates (mainly for monkeys) until it was first identified as a human pathogen in Malaysia in 1971 (Singh et al., 2004a). It was reclassified as a human pathogen by 2004 when more cases were identified (Singh et al., 2004a; Cox-Singh et al., 2008). In summary, it has now been established that human malaria is caused by five species of *Plasmodium* (*P. falciparum*, *P. knowlesi*, *P. malariae*, *P. ovale*, and *P. vivax*), of which *P. falciparum* is the most deadly form (Figure 1.1; WHO, 2014).
Figure 1.1 Classification of apicomplexans

Genus *Plasmodium* is grouped amongst apicomplexan class that is divided into four groups of coccidia, hematozoa, cryptosporidia and gregarine. The hematozoa group includes hemosporidiae and piroplasms. The piroplasms are subclassified into two genuses *Babesia* and *Theileria*. The hemosporidiae are subclassified into genuses *plasmodium* and *leucocytozoon*. The plasmodium genus is further subclassified into several species which are grouped according to host (*) represent human parasites (Adapted from Duval et al., 2007; Morrison, 2009; Janouškovec et al., 2010).
1.2.1 *P. falciparum* life cycle

Malaria infection occurs when an infected vector the female *Anopheles* mosquito, takes a blood meal from a host-human and inoculates the *Plasmodium* sporozoites into the blood (Figure 1.2; Koella *et al*., 1998). The sporozoites move in the blood circulation and subsequently infect hepatocytes (Lasonder *et al*., 2008). In the hepatocytes, initial asexual replication takes place and the parasites develop further into exo-erythrocytic shizonts, which rupture releasing merozoites into the blood stream where they infect red blood cells (RBC) (Bannister *et al*., 2000). Entry of the merozoites into the RBC is facilitated by parasite proteins which interact with RBC surface receptors (Boyle *et al*., 2010). The merozoites undergo asexual multiplication (erythrocytic-schizogony) in RBC which ruptures after 48 hours releasing 8-12 merozoites (Fujioka and Aikawa, 2002). The majority of the released merozoites infect more erythrocytes (Bannister and Mitchell, 2003).

![Figure 1.2 The Life cycle of *P. falciparum*](image)

Mosquito stage is characterized by the sporogonic cycle where a mosquito ingests the gametocytes during an infected blood meal (Fujioka and Akiwa, 2002). The Human liver stage starts where the sporozoite migrate to the liver and develop into schizonts. The erythrocytic cycle starts when the merozoites released form hepatocytes infect the RBC, adapted from Cowman *et al* (2012).
During the febrile episodes, some of the merozoites differentiate into sexual gametocytes; microgametocyte (male) and macrogametocyte (female) (Billker et al., 1997), which are ingested by *Anopheles* mosquito when they take a blood meal. The parasites in the mosquito multiply by sexual reproduction. The microgametocytes penetrate macrogametocytes in the mosquito stomach generating zygotes (Figure 1.2). Zygotes are motile and elongated (Ookinetes) and invade the midgut wall and develop into oocysts (Billker et al., 1997). The oocysts grow and rupture releasing sporozoites which migrate to the mosquito salivary gland epithelium and are inoculated into the host when the mosquito takes another blood meal and the cycle continues (Crompton et al., 2010).

The erythrocytic stages of the *Plasmodium* life cycle are responsible for the clinical manifestations of clinical malaria characterized by febrile episodes (Pavithra et al., 2004). The immune response to the antigenic parasite proteins stimulates release of proinflammatory cytokines among them tumour necrosis factor (TNF) which is responsible for periodic fevers that may elevate body temperature to 41°C (Bannister et al., 2000). It is at this crucial stage that the parasites asexually reproduce. During this stage there is increased protein biosynthesis mainly for aiding the rapid multiplication of the parasites.

### 1.2.2 Treatment and diagnosis

The effectiveness of therapeutic drugs in the control of malaria routinely faces challenges from drug resistant *P. falciparum* parasites. Global reports of *Plasmodium* strains resistant to quinolines, endoperoxidases and sulfadoxide-pyrimethamine drugs in malaria endemic areas have been documented (Witkowski et al., 2009; WHO, 2012). The development of the antimalarial drug Artemisinin derived from *Artemisa annua* plant, ushered in a new hope for the treatment of malaria (Yu, 2011). The promising drug, Artemisinin is also losing its efficacy as resistance to its monotherapy has been reported in the great Mekong sub region (Thailand, Cambodia, Myanmar and Vietnam) (Dordorp et al., 2009; Witkowsiki et al., 2012). To counter the development of drug resistance, treatment of malaria currently depends on Artemisinin based Combination Therapy (ACT) as the first line treatment endorsed by World Health
Assembly in 2007 (WHO, 2014). A recent publication reported a *P. falciparum* strain with reduced susceptibility to ACT in the Cambodia region and surrounding areas (van Schalkwyk and Sutherland, 2015). This poses a threat to the future efficacy of ACT with dire public health effects (van Schalkwyk and Sutherland, 2015). There is a high unmet clinical need for new *Plasmodium* drug targets to produce novel therapeutics against the increasing multidrug resistant strains. It is thought that the multidrug resistance observed in some *Plasmodium* strains is possibly facilitated by parasite targeted gene mutations (Su *et al*., 1997; Park *et al*., 2012). The ability of the parasite to thrive under drug selection stress may partly be facilitated by the molecular chaperones whose main function is to refold proteins including their mutated forms thereby enabling the parasite to resist drug pressure (section 1.4; Reviewed in Shonhai, 2010; Zinninga and Shonhai, 2014).

1.2.3 Malaria pathology

The pathology of *P. falciparum* malaria is mainly evident during the RBC stage of its life cycle (Le Roch *et al*., 2003). The parasites develop in the infected RBC (iRBC) resulting in their rupture. The intracellular multiplication of the parasite is associated with structural and biochemical changes that alter the functioning of the iRBCs. This leads to the development of the clinical symptoms of *P. falciparum* malaria which gives rise to anemia and/or cerebral malaria (Miller *et al*., 1994). Malaria pathology is influenced by host inflammatory reaction and parasite sequestration in tissues (Miller *et al*., 1994).

Sequestration malaria pathology is linked to iRBCs through their tendency to stick to both capillary endothelial cells and to uninfected RBCs leading to seclusion and rosette formation respectively (Martins and Daniel-Ribeiro, 2013). The iRBCs start showing morphological changes characterized by knob like structures that develop on their surface (Spycher *et al*., 2003). This normally occurs when the parasites reach the second half of the 48-hour maturation cycle. This is because at this stage the parasites export some of its own proteins to the iRBC membrane where they bind to receptors on endothelial cells that line the microvasculature (Xu *et al*., 2013; Saigal *et al*., 2014). The sequestration of iRBC is thought to
be facilitated by the binding of *P. falciparum* Erythrocyte Membrane Protein 1 (PfEMP1), present on the membrane of iRBC complexed to the endothelial receptors (Kraemer et al., 2006). The endothelial receptors include intracellular adhesion molecule 1 (ICAM-1) and thrombospondin (TSP) (Lucas and Sherman, 1998; Storm and Craig, 2014). The iRBCs can tether and roll on host receptors including ICAM1 and may enhance binding to platelet Glycoprotein IV or Cluster of Differentiation 36 (CD36) leading to sequestration (Reviewed in Angchaisuksiri, 2014). iRBCs are rigid, and this makes them to be sequestered in microvasculature (Dondorp et al., 2004). The rosetting and sequestration may lead to mechanical blockage of blood capillaries. These events are thought to result in obstruction of capillary blood flow leading to decreased tissue anoxia and decreased waste removal which may lead to a coma or organ failure (Dondorp et al., 2004; Martins and Daniel-Ribeiro, 2013).

The inflammatory pathway is based on host immune response when *P. falciparum* iRBC bursts, releasing both parasite toxins (glycosylphosphatidylinositol, hemozoin) and RBC intracellular molecules (heme) into circulation (Ropert et al., 2001; Nebl et al., 2005; Martins and Daniel-Ribeiro, 2013). These toxins are recognized by CD1 receptors on monocytes, neutrophils and endothelial cells through Toll-like receptor 2 (TLR2) pattern recognition receptors (Nebl et al., 2005). Upon exposure to the toxins the CD1+ (with CD1 receptors) cells are activated and induced to secrete pro-inflammatory cytokines like tumor necrosis factor alpha (TNF-α), Interferon gamma (IFN-γ) and lymphotoxin-alpha (LT-α) (Nebl, 2005; van der Hyde et al., 2006). The activated innate immune cells together with released pro-inflammatory cytokines recruit CD4+ and CD8+ cells which further aggravate the inflammation by recruiting more cells to secrete pro-inflammatory cytokines (van der Hyde et al., 2006). The inflammation is beneficial at first, in reducing the parasitemia and the release of toxic intracellular molecules. However, later on, the inflammatory response is not properly regulated and consequently mediates damage to the microvascular endothelium resulting in leakage of cytokines, malarial antigens and free radicals resulting in the aggravation of the disease (Hunt et al., 2006). Taken together, sequestration and inflammation pathways are complimentary of each other as both are based on processes that occur during *P. falciparum*
infection. Altogether, these phenomena explain why malaria pathology is multifaceted as it involves both host and parasite factors.

1.2.4 Parasite-host interaction

The invasion of RBC by a parasite is a process that can be described in three distinct stages: (i) attachment with apical reorientation followed by (ii) induction of the parasitophorous vacuole (PV) (Figure 1.3) and subsequently (iii) translocation of parasite into the vacuole. The parasite attaches to the host RBC membrane using the apical end contacts that interact with cell receptors which stimulate parasite reorientation (Baumeister et al., 2010). Reorientation involves the gliding motility mediated by the glideosome, an actinomyosin-based protein complex (Baumeister et al., 2010). The reorientation results in direct juxta-positioning of parasite apical end and host cell membrane resulting in the formation of a tight junction between the parasite apex and the host RBC (Cowman and Crabb, 2006). This induces the secretion of adhesins from the micronemes that are translocated along the parasite length and are shed on the side of the moving junction (Richard et al., 2010). The secretion of PV components from the rhoptries also plays an important role in this event (Baumeister et al., 2010; Richard et al., 2010). The PV is a compartment that is formed in a circumferential zone of attachment of the orifice of the host cell invagination. The parasite enters the nascent PV by capping the moving junction down its body thought to be facilitated by the *P. falciparum* merozoite-capping protein 1 (MCP-1) (Cowman and Crabb, 2006). The parasite becomes enclosed within the PV (cavity) delimited by the invaginated host membrane (Figure 1.3; Baumeister et al., 2010).

The PV is surrounded by the parasitophorous vacuole membrane (PVM) formed during invasion. The PV serves as an interface between the parasite and host iRBC cytosol (Baumeister et al., 2010). The PVM has been shown to lack the RBC membrane proteins like glycophorins and anion transporter band 3 (Baumeister et al., 2010). This suggests there is selective recruitment of cholesterol-rich sub-domains within the RBC membrane called lipid “rafts” associated membrane proteins (Figure 1.3; Harrison et al., 2003; Baumeister et al.,
The recruited erythrocyte proteins associated with the PVM form detergent resistant micro domains which serve as a shield to protect the parasite (Tokumatsu et al., 2014).

Figure 1.3 Representation of a cross section of the parasite infected erythrocyte
The letters represent the following organelles; ER (endoplamic reticulum), Mt (Mitochondria), AP (Apicoplast), PV (Parasitophorous vacuole), TVN (Tubulovesicular network) and PVM (PV membrane), adapted from Shonhai et al (2007).

The transportation of more than 400 parasite proteins beyond the PVM is thought to be mediated by a pentapeptide signal located towards the N-terminus of target proteins (Hiller et al., 2004), termed Plasmodium export element (PEXEL) (Marti et al., 2005) or the vacuolar transport signal (VTS) (Hiller et al., 2004). A second group of exported proteins lack the PEXEL motif and they are termed PEXEL-negative exported proteins (PNEP) (Grüning et al., 2012). For PNEPs their export is thought to be mediated through a single hydrophobic region that is located up to 214 amino acids downstream from the N-terminal (Spielmann et al., 2006; Spielmann and Gilberger, 2010). The export of both PEXEL and PNEPs is thought to be facilitated by unfolding of proteins thereby suggesting translocation as a common step in export (Gruring et al., 2012). There are suggestions that a multimeric protein complex translocon located on the PVM is responsible for the traffic of parasite proteins (Charpian and Pryzborski, 2008). The PVM has non-selective pores that facilitate the transport of nutrients to the parasite (Figure 1.3; Nyalwidhe et al., 2002).
The exported parasite proteins that mainly associate with the RBC membranes are the ring-infected erythrocyte surface antigen (RESA) (Goel et al., 2014), mature-parasite-infected erythrocyte surface antigen (MESA) (Kilili and LaCount, 2011), *P. falciparum* erythrocyte membrane protein 3 (PfEMP-3) (Pei et al., 2007), knob-associated histidine-rich protein (KAHRP) (Weng et al., 2014) and *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) (Senczuk et al., 2001). Amongst these KAHRP and membrane-associated histidine-rich proteins 1 (MAHRP) (Spycher et al., 2003) associate beneath the knobs (Spycher et al., 2003; Pei et al., 2007; Weng et al., 2014). The Maurer’s clefts are discrete membrane bound appendages that are scattered in the iRBC cytoplasm where they mediate parasite protein assembly (Figure 1.3; Cooke et al., 2006). *P. falciparum* skeleton-binding protein 1 (PfSBP1) (Kats et al., 2015) and MAHRP are thought to be localized in the Maurer’s clefts. RESA is expressed at the ring stage and is thought to contribute to the RBC membrane structural integrity through binding to beta spectrin (Pei et al., 2007). MESA facilitates binding of protein 4.1 to glycophorin C, band 3 and p55 (Waller et al., 2003; Pei et al., 2007). KAHRP is involved in the adhesive interaction of the merozoite to the RBC membrane through binding to the repeat 4 of alpha spectrin (Weng et al., 2014). The parasites remodel the RBC as a survival mechanism to evade clearance by the immune system. PFEMP-1 is also implicated in RBC cytoadherence to prevent spleen clearance and also it is implicated in antigenic diversity as a mechanism of immune system evasion (Biggs et al., 1991; Buffet et al., 2011; Merrick et al., 2012).

### 1.2.5 Red blood cell modification by *P. falciparum*

*P. falciparum* infected RBCs have been found to exhibit increased phosphorylated protein Band 4.1 (Morrissette and Sibley, 2002). Parasite cysteine protease cleaves the iRBC ankyrin and this results in loss of the normal discoid shape of the RBC to spherical orientation (Raphael et al., 2000). These modifications together with other exported parasite proteins RESA, MESA and histidine rich protein 1 (HRP-1) (Hayward et al., 2002) which are anchored on the RBC membrane associate with spectrin, band 4.1 and band 3 ankyrin binding domain. This association results in altered RBC morphology, a mechanism used by parasites for immune evasion. The integrity of the iRBC cytoskeleton is crucial for parasite development
(Morrissette and Sibley, 2002). This suggests that the parasite survives indirectly from structural features of the iRBC. At the end of 48 hour asexual cycle, the iRBC membrane is ruptured. This process is thought to be initiated by disruption of the PV and the entry of parasites into the RBC cytoplasm (Morrissette and Sibley, 2002; Mohandas and An, 2012). Parasite serine proteases mediate the destabilization of the iRBC membrane culminating in its rupture releasing new merozoites (reviewed in Mohandas and An, 2012).

1.3 Molecular Chaperones

1.3.1 Heat shock proteins as molecular chaperones

Molecular chaperones are molecules that are involved in the folding of nascent polypeptides, refolding of unfolded proteins, and facilitation of protein transport across membranes, reduce aggregation and facilitate degradation of clients that are beyond refolding as a quality control mechanism (Bukau et al., 2006). Molecular chaperones facilitate non-covalent three-dimensional organization of their clients without themselves taking part in the final product.

Heat shock proteins (Hsp) are a group of molecular chaperones, most of which are upregulated in response to stress. Heat shock proteins were named upon their first discovery in Drosophila where they were upregulated during heat stress (Ritosa, 1962). They are involved in the suppression of stress induced unfolding of client proteins and also refolding of unfolded proteins. Some Hsps have the ability to fold newly formed proteins. Hsps play a central role in proteostasis as they are also involved in determining the fate of misfolded proteins either refolding them or channeling them to the ubiquitin proteolysis pathway for degradation (Kriegenburg et al., 2012).

Hsps are ubiquitous molecular chaperones found in all celled organisms. Hsps exhibit high level of conservation across species. Despite the fact that heat shock proteins constitute the bulk of molecular chaperones, not all molecular chaperones are heat shock proteins. There are several ways in which Hsps are named, but generally those that are stress induced are
referred to as Hsp, and those that are cognatively expressed are termed heat shock cognate (Hsc) proteins. They can also be named according to their functional roles; holding substrate in a folding competent state (holdase), folding the substrate to its native structure (foldase) (Wyn et al., 1994) and unfolding aggregated substrates (unfoldase) (Saibil, 2013). Hsps are also classified according to their molecular sizes (Table 1.1; Lindquist and Craig, 1988).

1.4 The Role of Heat Shock Proteins in the Development of *P. falciparum* parasites

1.4.1 Major *P. falciparum* heat shock proteins

There are 7 major families of heat shock proteins from *P. falciparum* denoted according to size; Hsp110, Hsp100, Hsp90, Hsp70, Hsp60, Hsp40 and small Hsps (approximately 15-30 kDa) (Table 1.1).
Table 1.1: Major shock proteins families and their cellular localisations

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Monomer size/ kDa</th>
<th>Eukaryotic localisation</th>
<th>Stress function</th>
<th>Comments</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hsp110</td>
<td>100-150</td>
<td>cytoplasm ER</td>
<td>nucleotide exchange factors, thermotolerance</td>
<td>function as NEFs for Hsp70s, have independent holdase activity</td>
<td>Oh et al (1999); Dragovic et al (2006); Muralidharan et al (2012)</td>
</tr>
<tr>
<td>Hsp70</td>
<td>67-76</td>
<td>ER, cytosol, nucleus, mitochondria, chloroplast</td>
<td>chaperone required for protein assembly, secretion, thermotolerance</td>
<td>associates with; Hsp60, Hsp40, Hsp90,GrpE; binds unfolded proteins and peptides; an ATPase (foldase)</td>
<td>Kurtz et al (1986); Bukau and Walker (1990); Shonhai et al (2005)</td>
</tr>
</tbody>
</table>

Table legend: ER- endoplasmic reticulum; BiP- binding protein; Grp- glucose-regulated protein; Hsp- heat-shock protein; Clp- Caseinolytic protease, adapted from Lindquist (1992).

1.4.2 Hsp100 family

Hsp100 group of molecular chaperones (Table 1.1) are primarily involved in the dis-assembly of quaternary structure of polypeptide complexes and are crucial elements required for thermotolerance (Sanchez and Lindquist 1990; Pak and Wickner, 1997). Hsp100s, specifically Hsp104 and prokaryotic ClpB (caseinolytic protease B) structurally have two nucleotide binding domains (NBD1 and NBD2; AAA+ domains) which are important for chaperone
function and form functional hexameric complexes (Vale, 2000; Nowicki et al., 2012). The first AAA+ domain of Hsp104 and ClpB contains an additional coil-coil region called the middle domain located on the C-terminus of the ATP binding site (Winkler et al., 2012; Mogk et al., 2015). Eukaryotic and prokaryotic Hsp100 utilizes an ATP hydrolysis functional cycle which is thought to be stimulated by substrate binding (Nowicki et al. 2012). The N-terminus serves as the substrate binding domain (Barnet et al., 2005). The function of Hsp100 as a disaggregase and ATPase are modulated through a conserved aspartic acid residue D184 in the NBD1 (Hsp104), analogous to ClpB (D178) which is required for both ATPase and dis-aggregation functions (Nowicki et al., 2012). This suggests that Hsp100 have a conserved switch that modulates both the ATPase and disaggregase function.

Hsp100 functions in a complex with Hsp70s to form Hsp100/Hsp70 disaggregate complex that entangles polypeptides from mesh of aggregates (Winkler et al., 2012; Mogk et al., 2015). In this Hsp100/Hsp70 complex, it has been postulated that Hsp100 is the first one to bind to aggregated substrates, pulling them out from the aggregated mesh thereby exposing the hydrophobic patches where Hsp70 subsequently binds allowing the substrate to fold into its native form (Mogk et al., 1999; 2015).

P. falciparum encodes eight Clp proteins; five ClpATPases that belong to the AAA+ superfamily (PfClpB1, PfClpB2, PfClpC, PfClpM, PfClpY; El Bakkouri et al., 2010; Rathore et al., 2011), and three protease like proteins: PfClpP, PfClpR and PfClpQ (El Bakkouri et al., 2010; 2013). The Clp AAA+ proteins are localized in the apicoplast with the exception of PfClpB2 (PV) and PfClpY (Mitochondia; El Bakkouri et al., 2010). ClpB was shown to be localized in the PV and is thought to facilitate secretion of malarial proteins into the PV (Rathore et al., 2011). Clp proteins have also been shown to function as heptemer complexes, whose function is essential in apicoplast development (Rathore et al., 2010). There is need for further studies to elucidate possible interactions between Clp ATPases and P. falciparum Hsp70s (PfHsp70-3 localized in the mitochondrial and PfHsp70-x in the PV (section 1.4.6.3 and 1.4.6.4, respectively) as this may suggest the formation of functional complexes with an important role in parasite proteostasis.
1.4.3 Hsp90 family

Heat shock protein 90s (Hsp90) (Table 1.1) are ATP dependent molecular chaperones involved in a variety of cellular processes including cell cycle control, cell survival, hormone and other cell signaling pathways (Jackson, 2013). Hsp90s are widespread in eubacteria and in eukaryotes. They are the most abundant molecules, for example, in the human cell they constitute about 2% of the total cell protein mass (Finka and Goloubinoff, 2013; Finka et al., 2015). As such, Hsp90s have been used as drug targets in neurodegenerative disorders, cancer, and in the development of anti-viral and anti/protozoan infections (reviewed in Zininga and Shonhai, 2014). Hsp90 recognizes substrates from a wide range of protein families (Jackson, 2013). In S. cerevisae it was shown that at least 10% of the proteins require Hsp90 for further folding (Zhao et al., 2005).

The Hsp90 family is highly conserved from eubacteria to eukaryotes (Johnson and Brown, 2009). For example, Hsp90 from human and E. coli share ~50% sequence identities (Johnson and Brown 2009; Johnson, 2012). Hsp90 chaperones are essential in eukaryotes but are not essential at all growth conditions in E. coli (Cao et al., 2003; Maynard et al., 2010). Hsp90 family is composed of five subfamilies in different cellular locations: cytosolic Hsp90s, ER Hsp90s (Grp94), Mitochondrial TRAP1, chloroplast Hsp90 and bacterial Hsp90 HtpG (Jackson et al., 2013). In mammals there are two cytosolic Hsp90s; the constitutively expressed Hsp90β and the inducible isoform Hsp90α (Kampinga et al., 2009). These function as homodimers that interact through the C-terminal domain (Figure 1.4; Jackson et al., 2013).

Hsp90 are composed of an N-terminal domain that serves as the ATP binding site and is linked to the middle domain through a charged linker. The middle domain is adjacent to the C-terminal domain which ends with an EEVD motif at the C-terminal (Figure 1.4). In P. falciparum there are four Hsp90 paralogs; full length cytosolic Hsp90 homologue (PF3D7_0708400), endoplasmic reticulum Grp94 homologue (PF3D7_1222300), mitochondrial TRAP1 (PF3D7_1118200) and a truncated Hsp90 without the cytosolic signal
(PF3D7_1443900) (Kumar et al., 2003; Acharya et al., 2007). Similar to the other Hsp90s, they possess the EEVD motif towards the C-terminus (Banumathy et al., 2003; Gitau et al., 2012).

Figure 1.4 Structure and mechanism of Hsp90 homodimer
The NTD is N-terminal domain that has ATPase activity (Prodromou et al., 1997) which also bind ATP analogues like geldanamycin (Roe et al., 1999), followed by a charged linker that is absent in HtpG. The charged linker can modulate the activity of Hsp90 (Tsutsumi et al., 2012) The middle domain (MD) contains a catalytic arginine that is required for ATP hydrolysis, binds to Aha co-chaperones and is thought to be the main client binding domain, (CTD) the C-terminal domain contains the major dimerization interface that makes Hsp90 to constitutively function as homodimers. At the c-terminal there is a conserved MEEVD motif that binds to TPR domains on co-chaperones including Hop/Sti1, FKBP51/52, Cyp40.

Despite the fact that other Hsp90 homologues have low basal ATPase activity, *P. falciparum* Hsp90 (PfHsp90) has hyper-active basal ATPase activity (Pallavi et al., 2010). This activity is sufficient to induce conformation changes resulting in substrate release (Pallavi et al., 2010; Krukenberg et al., 2011). The high ATPase activity of PfHsp90 imparts the protein with a high substrate turnover as compared to its human homologue (Krukenberg et al., 2011). PfHsp90 ATPase domain can be specifically targeted by Geldanamycin and its derivative 17-allylaminogeldanamycin (17-AAG) which inhibits the activity of the chaperone (Pallavi et al., 2010). In *P. falciparum* Hsp90 is an essential molecule (Pallavi et al., 2010) and this explains why Hsp90 is targeted as a drug candidate (Zininga and Shonhai, 2014).
The Hsp90 chaperone function is mediated by the assistance of other chaperones and co-chaperones. Hsp90 interacts with Hsp70 (section 1.4.6), to facilitate substrate transfer for further folding (Scheufler et al. 2000). This interaction is modulated by Hsp70-Hsp90 organizing protein (Hop/Sti1), which functions as an adaptor protein linking Hsp90 and Hsp70 (Nicolet and Craig, 1989). Hop is characterized by the presence of tetratricopeptide repeat (TPR) domains, TPR1 and TPR2A motifs (Scheufler et al. 2000). Both Hsp90 and Hsp70 possess the EEVD motifs towards the C-terminus which are required for interaction with TPR domains on Hop co-chaperone (Banumathy et al., 2003; Gitau et al., 2012; Schmid et al., 2012). The mechanism of action of Hop has been proposed in two distinct ways: some postulate that Hsp70 binds to substrate first and then interacts with Hop/Sti1 and passes the substrates to Hsp90 for further refolding (Li et al., 2013; Röhl et al., 2015). Hsp90 exhibits holdase chaperone function as it has the ability to recognize and hold nonnative substrates preventing their aggregation (Weich et al., 1992).

A P. falciparum Hop homology has been described and it is thought to mediate the interaction between PfHsp90 and PfHsp70-1 complex similar to that of mammalian Hsp90 (Banumathy et al., 2003; Gitau et al., 2012, Röhl et al., 2015). The interaction of Hop/Sti1 and Hsp90/Hsp70 is through its multiple tetratrico-peptide repeat domains (TPR1, 2A and 2B) which interact with the C-terminal EEVD motif on both Hsp90 and Hsp70 (Scheufler et al., 2000; Gitau et al., 2012; Schmid et al., 2012). Hsp90 facilitates the Hsp90/Hop/Hsp70 complex formation and substrate handover (Röhl et al., 2015). Binding of Hsp90 on TPR2A causes conformational changes on TPR2B with subsequent increase in the affinity of TPR2B for Hsp70 (Röhl et al., 2015). This results in Hsp70 bound on TPR1 shifting and binding to TPR2B when Hsp90 is bound to TPR2A (Röhl et al., 2015). Altogether, this suggests that Hsp70 substrate complex binds to Hop TPR1 first, then when Hsp90 is docked on TPR2A subdomain Hsp70 moves to TPR2B during which time it passes the substrate to Hsp90.
1.4.4 Hsp60 family

The Hsp60 family is mainly composed of two essential prominent proteins the GroEL (chaperonin 60)/GroES (chaperonin 10) in prokaryotes (Table 1.1; Fayet et al., 1989; Viitanen et al., 1990) and the t-complex polypeptide-1 (TCP-1 complex) (McCallum et al., 2000). TCP-1 is structurally comprised of a heterocomplex which mainly facilitates the assembly of actin and tubulin (McCallum et al., 2000). TCP-1 closely associates with client polypeptides coming off from the ribosomes. The GroEL are involved in the assembly of multi-protein complexes by forming a central cavity where substrates are isolated (Ellis, 1994). The central cavity acts as an isolation chamber where GroES acts as the lid which closes the central cavity forming the Anfinsen cage (Ellis, 1994; Houry et al., 1999). This results in the formation of a double ring composed of 14 subunits whose central cavity binds to substrates through hydrophobic interactions (Ellis, 1994). Each subunit of GroEL is subdivided into three domains, apical; equatorial and the contact for ring binding. The apical domain has the substrate and GroES binding sites and the equatorial domain has ATP binding site (Houry et al., 1999; Inobe et al., 2008). The apical and equatorial domains are connected by an intermediate subdomain, which acts as a hinge facilitating conformational changes induced by ATP binding (on the equatorial domain) (Villas et al., 2014). The conformational changes on the substrate binding surface determine either the surface hydropathy index (indicator of hydrophobicity or hydrophilicity). Substrate binding occurs when TCP-1 is in hydrophobic state. Thus the first ATP binding results in higher affinity (hydrophobic state) for substrate binding and the binding of the subsequent ATP results in an opposite effect of substrate release when it changes to the hydrophilic state (Ueno et al., 2004; Inobe et al., 2008).

The *P. falciparum* genome encodes for two Hsp60 GroEL like members, PF3D7_123100 (PfCpn60) and PF3D7_1015600 (PfHsp60) localized in the apicoplast and mitochondria, respectively (Sato and Wilson, 2004; Sato et al., 2005). The *P. falciparum* genome also encodes for GroES homologues PF3D7_1333000 (PfCpn20) and PF3D7_1215300 (PfCpn10) localized in the apicoplast and mitochondria respectively (Sato and Wilson, 2005). The co-localisation of these Hsp60 chaperonins (PfCpn60 with PfCpn20 in the apicoplast) and
(PfHsp60 with PfClpn10 in mitochondria) suggests the possibility of a functional complex in the apicoplast and mitochondria.

1.4.5 Hsp40 family

Heat shock protein 40 (Hsp40) family members are approximately 40 kDa in size (Table 1.1). In prokaryotes they are referred to as DnaJ based on the presence of the highly conserved J-domain comprising of approximately 70 amino acids (Laufen et al., 1999). There is a variation in the number of Hsp40s in different organisms. They are generally fewer in prokaryotes (E. coli, 6; Synechococchus elongates, 3; Qiu et al., 2006; Kampinga et al., 2009). Eukaryotes have expanded Hsp40s members, for example, humans have at least 50; S. cerevisiae have at least 22 and at least 49 in P. falciparum (Kampinga et al., 2009; Njunge et al., 2013).

Generally, Hsp40s are grouped into four types (I-IV) mainly based on their structural and functional characteristics (Cheetham and Caplan, 1998; Botha et al., 2011; Rug and Maier, 2011). Hsp40s can be classified structurally based on the presence of three typical domains: a J-domain with or without a conserved tripeptide of His, Pro and Asp (HPD) motif; the glycine–phenylalanine (GF) rich region and cysteine rich domain (CRR) (Figure 1.5; Njunge et al., 2013). Type I Hsp40s are composed of a J-domain (with a HPD motif); GF region and CRR (Figure 1.5 Botha et al., 2007). Type II Hsp40s contain the J-domain (with HPD motif) and the GF region two but lack the CRR (Figure 1.5; Botha et al., 2007). Type III Hsp40s are composed of the J-domain with the HPD motif but they lack both the GF rich region and the CRR (Figure 1.5). Type IV contains the J domain (without the HPD motif) whose position is variable and they lack the GF rich region and the CRR (Figure 1.5; Botha et al., 2007).

Type I and type II Hsp40s have the capability to bind substrate through the CRR and the C-terminal region (Figure 1.5; Botha et al., 2007; 2011), but they cannot refold the substrates. Type I and type II are capable of suppressing aggregation (Botha et al., 2011). Type I and Type II Hsp40s functionally associate with Hsp70s to activate its ATPase (Njunge et al., 2015). Their interaction with Hsp70s could be described in two distinct functional features: substrate
recruitment where they bind and bring substrates to Hsp70s and also as Hsp70 ATPase activators (Horne et al., 2010; Bascos and Landry, 2015; Njunge et al., 2015). The interaction between Hsp70 and Hsp40 is mediated through the J domain of the Hsp40 (Bascos and Landry, 2015).

The GF-rich region on Hsp40s is useful for regulation of Hsp70 substrate binding (Walsh et al., 2004). Type I and type II Hsp40s bind substrates not only for recruitment to Hsp70 but they also suppress thermally induced misfolding of substrates and assist in multi-complex protein formation (Ansorge et al., 1996; Fan et al., 2003). Type I and type II, Hsp40s have been shown to interact with Hsp70 in the chaperone cycle involving progesterone receptor (Pratt and Toft, 2003; Young et al., 2004). Type I Hsp40; Ydj1 (yeast) and HDj2 (human) bind to substrate with tight stable affinity, whilst type II, HDj1 (human) bind with rapid on/off rates (Cintron and Toft, 2006). This suggests the cysteine rich region (CRR) on type I Hsp40s stabilizes substrate interaction.
Type III Hsp40s are functionally distinct from type I and type II in that they don’t seem to bind non-native substrates, although they present themselves at the Hsp70 site of action (Xiao et al., 2006). For example, yeast type III, Swap2p and its mammalian homology axullin, present themselves at the site of action by associating with Hsp70 during uncoating of clathrin-coated vesicles (Xiao et al., 2006). Type IV was first described by Botha and colleagues (2007).

Of the 49 members in *P. falciparum* there are two type I Hsp40s, eight type II, twenty-six type III and thirteen type IV (Njunge et al., 2013). Nineteen of Hsp40s have the PEXEL sequence, suggesting that they are exported to the iRBC cytosol where they possibly participate in host cell remodeling and interaction with host cell cytoskeleton (section 1.24; 1.2.5; Archarya et al., 2012; Njunge et al., 2013). This implicates Hsp40s as central chaperones playing an important role during iRBC cell remodeling which characterizes malaria pathology. Some exported type IV Hsp40s have been shown to be essential for parasite survival and pathogenesis for example, PF11_0034 (PF3D7_1102200), RESA (PF3D7_0102200) and PF10_0382 (PF3D7_1039100; Maier et al., 2008; Njunge et al., 2013).

Comparative functional assays conducted by Botha and colleagues (2011) showed possible functional conservation of *P. falciparum* Hsp40s type 1 with their human (HspA1A) and yeast (DnaJA1) equivalents. Interestingly, biochemical assays have also shown that a type I PfHsp40 (PF3D7_1437900) interacts with PfHsp70-1 (section 1.4.6.5.1) forming a functional PfHsp70-1-PfHsp40 complex (Botha et al., 2007). This functional complex promoted PfHsp70-1 ATPase hydrolysis. Furthermore, there is evidence of co-localization of PfHsp70-1 and PfHsp40 within the parasite cytoplasm (Botha et al., 2011). It should be noted that Hsp70-Hsp40 partnerships tend to exhibit functional specificity across species. PfHsp70-1 and PfHsp40 complex was differentially inhibited with Hsp70 inhibitor pyrimidinone when compared to human (HspA1A) and yeast (DnaJA1) counterparts showing selective inhibition (Botha et al., 2011). This suggests that *P. falciparum* Hsp40s can be specifically inhibited and it makes them potential drug targets (Botha et al., 2011; Cockburn et al., 2011; Zininga and Shonhai, 2014).
1.4.6 Hsp70 family

Hsp70 is one of the most evolutionary conserved superfamily of Hsps that occur in all domains of life archaea, eubacteria and eukaryotes (Table 1.1; Lindquist and Craig 1988). In archaea and eubacteria Hsp70s, are referred to as DnaK. In yeasts, they are named as Ssa, in mammals including humans they are named as HSPA (Kampinga et al., 2009). Hsp70 is one of the most prominent molecular chaperones required for several cellular activities, which include folding of nascent polypeptides (Szabo et al., 1994), regulation of kinases (Asea et al., 2002), denaturation of protein (Bercovich et al., 1997), trafficking of proteins (Gambill et al., 1993), protein translocation, assembly and disassembly of oligomeric complexes (Lüders et al. 2000; Mayer and Bukau, 2005; Mayer, 2010). Hsp70s are ubiquitous molecules, and some of their homologues are stress inducible. Furthermore, canonical Hsp70s (DnaK like) are capable of both suppressing protein aggregation and refolding misfolded peptides (Kurt et al., 2006; Mayer, 2013).

Hsp70 are generally composed of an N-terminal 44 kDa-Nucleotide binding domain (NBD) (Hsp70\textsubscript{NBD}) with ATPase activity, substrate binding domain (SBD) (Hsp70\textsubscript{SBD}) and a C-terminal lid (Figure 1.6). There is high sequence conservation on the Hsp70\textsubscript{NBD} and the Hsp70\textsubscript{SBD} is less conserved (Easton et al., 2000). These two domains are connected by a highly conserved 7-residue linker region (Sharma and Masison, 2009). Hsp70s are divided into two sub-families: DnaK-like (canonical Hsp70s) and Hsp110 (Figure 1.6; Section 1.4.6.2.5; Easton et al., 2000). DnaK is capable of refolding misfolded protein and suppressing protein aggregation (Mogk et al., 1999). DnaK and DnaK-like Hsp70s from other species are considered to be canonical Hsp70s. On the other hand, Hsp110 members are specialized proteins that are structurally distinct from canonical Hsp70s. They have been described as nucleotide exchange factors (NEF) of canonical Hsp70s (Dragovic et al., 2006; Andreasson et al., 2008).
Hsp70 domains are connected by a highly conserved linker segment whose mutation leads to functional abrogation, suggesting that inter-domain communication of Hsp70 is crucial for their function (Vogel, 2006). The linker motif of canonical Hsp70s is composed of the residues, DVLLLDV (Easton et al., 2000; Vogel, 2006). Allosteric signals from the linker are sent in two directions; binding of nucleotide to the N-terminal NBD is followed by transmission of the signal to the SBD via the linker segment; and similarly binding of misfolded peptide to the SBD alters the conformation of NBD and subsequently the rate of ATP hydrolysis (Zhuravleva and Gierasch, 2011).

1.4.6.1 Hsp70 substrate binding

Hsp70s possess a highly conserved NBD which is related to actin and is composed of four domains IA, IB, IIA, and IIB. These are arranged in two lobes that are linked and create a crevice for nucleotide binding (Figure 1.6B). The crevice has a rather hydrophilic pocket that specifically binds to the adenosine and the phosphates on ATP. Hsp70_{SBD} is composed of a unique two layered twisted β-sandwich (SBDβ) that has a hydrophilic cleft where the
hydrophobic patches on substrates are bound (Flaherty et al., 1990; Kityk et al., 2012). The helical SBD\(\alpha\) ends with a rather unstructured stretch of about 30 residues (Flaherty et al., 1990). X-ray crystallographic studies have shown that in the open conformation the two SBD domains (SBD\(\alpha/\beta\)) are detached from each other and both are anchored onto the different faces of the NBD (Kityk et al., 2012; Zhang et al., 2014). The association of the SBD\(\alpha\) and SBD\(\beta\) in the closed conformation is controlled by allostery and substrate binding (Kityk et al., 2012; Zhang et al., 2014).

In the closed conformation of Hsp70\(_{\text{SBD}}\) SBD\(\alpha\) acts as the lid to SBD\(\beta\) and promotes the stable binding of substrate (Figure 1.6B; Kityk et al., 2012; Mayer et al., 2013). The poly-peptide binding cleft contacts hydrophobic patches comprised of 7-8 residues of the bound peptide mostly through hydrophobic interactions (Kityk et al., 2012; Mayer, 2013). Hsp70 interacts with substrates from different sources. For example, it interacts with nascent polypeptides, polypeptides in complex formation, misfolded polypeptides and polypeptides during transport (Mayer, 2013). This versatility in binding is achieved through the recognition and binding of degenerative motif consisting of a core with five residues which are highly hydrophobic and flanked by positively charged amino acids (Mayer and Bukau, 2005). These motifs are known to occur after every 30 - 40 residues in virtually all proteins (Mayer, 2013). These hydrophobic patches are only exposed in newly synthesized polypeptide awaiting de novo folding and in denatured or unfolded proteins (Mayer and Bukau, 2005).

Substrate binding on Hsp70\(_{\text{SBD}}\) is specifically achieved through the center of the hydrophobic pocket constituted by five residues. The residues implicated are located on the SBD; \(\beta 1, \beta 3\) and \(\beta 4\) which bind a single substrate side chain (Figure 1.7; Mayer, 2013). The backbone of the substrate side chain is enclosed by strands 1 and 2 including loop L\(_{1,2}\) and the upward protruding loop L\(_{3,4}\). These form hydrogen bonds and hydrophobic interactions with the substrate (Kityk et al., 2012). Substrate interaction is further stabilized by a layer of Loops L\(_{4,5}\) and L\(_{5,6}\) (Kityk et al., 2012; Mayer, 2013). The vicinity of the peptide binding cleft has a net negative charge which is consistent with the observation that the substrate binding patch is
a hydrophobic structure that is flanked by positively charged residues (Kityk \textit{et al}., 2012; Mayer, 2013).

![Figure 1.7: Hsp70 substrate binding domain complexes with ATP](image)

Substrate enclosing loops are more distant from the substrate binding pocket of human Hsp70 (1DKX, Zhu \textit{et al} 1996) the loops are numbered from the N-terminal towards C-terminal (1-8), adapted from Kityk \textit{et al} (2012).

Upon ATP binding, Hsp70s undergo structural changes which register as a rotation on NBD lobe I relative to lobe II resulting in closure of the nucleotide binding crevice (Liu and Hendrickson, 2006). This results in opening of the lower lobes IA and IIA allowing their binding to the conserved inter-domain linker which results in ATP stimulated peptide release from the SBD and ATP hydrolysis (Vogel \textit{et al}., 2006; Kumar \textit{et al}., 2011; Kityk \textit{et al}., 2012). Peptide release is achieved through the effects of the rotation of the lobe on the NBD which displaces two residues by about 2 Å in the SBD catalytic center, Lys70 and Glu171 (numbering according to \textit{E. coli} DnaK) (Kityk \textit{et al}., 2012). Lys70 coordinates the \(\gamma\)-phosphate in the ATP bound state which stabilizes the pentavalent transition of the phosphate (Kityk \textit{et al}., 2012). This is then disrupted by Glu171 which together with Thr199 position water for the attack on the \(\gamma\)-phosphate (Kityk \textit{et al}., 2012; Mayer, 2013). This is the reason why ATP binding causes lobe rotation on the NBD which results in docking of the SBD. The docking of SBD on NBD lowers affinity for substrate resulting in substrate release (Mayer, 2013).
1.4.6.2 Hsp40-Hsp70 partnership

Hsp70 (section 1.4.6.1) has low basal ATPase activity where one molecule of ATP is hydrolyzed by one Hsp70 protein in ~25 minutes at 30 °C (Mayer et al., 2000; Alderson et al., 2014). This somehow slow activity has been shown to be activated by the binding of a substrate in the Hsp70\textsubscript{SBD} (Mayer et al., 2000). Hsp70 has lower affinity for substrates in its ATP bound state (section 1.4.6.1), then the synergy of bound substrate and Hsp40, results in up to 1000 fold ATP hydrolysis up-regulation compared to basal Hsp70 activity (Silberg et al., 2000; Kampinga and Craig, 2010; Needham et al., 2015). Hsp70 in the ADP bound state has higher affinity for substrates and exhibits longer substrate dwelling time.

Hsp40s are thought to strategically position themselves where client polypeptides are coming from. For example, at the ribosomal exit tunnel or the translocation pore, where they then bind client polypeptides and pass them over to Hsp70 for binding and subsequent folding (Kampinga and Craig, 2010). In this case, they act as ‘targeting factors’ for Hsp70 clients (Kampinga and Craig, 2010). Interestingly, Hsp40s are also thought to be responsible for recruiting Hsp70 to sites of protein aggregates. This role is essential with respect to the function of Hsp70 as a disaggregase (Torrente and Shorter, 2013). Upon its recruitment to the site (location of aggregate), Hsp70 subsequently partners with Hsp100 (section 1.4.2), in disentangling the aggregated polypeptides ultimately facilitating their refolding (Mogk et al., 2015). This is one hallmark of the function of Hsp70 in reducing toxicity of aggregates as in the case of neurodegenerative diseases which are characterized by accumulation of toxic aggregates (Voss et al., 2012). Hsp70s are also involved in the suppression of toxicity induced by aggregated reactive proteins. In this case, Hsp70 diffuses the aberrant accumulation of hyper-phosphorylated microtubules associated protein \textit{tau} called \textit{tau-tangles} (Voss et al., 2012), whose accumulation results in central nervous system (CNS) disorders such as Alzheimer’s disease and Pick’s disease (Sergeant et al., 2005).
1.4.6.3 The functional cycle of Hsp70s

The functional cycle of Hsp70s is known to be driven by ATP hydrolysis (Figure 1.8; Patury et al., 2009). The switches between ATP- and ADP bound states of Hsp70 regulates the off and on rates of peptide binding (Buchberger et al., 1995). In the ATP bound state Hsp70 has fast on-off rates resulting in low affinity for substrate and in the ADP bound state there is slow on-off rates that results in overall high affinity for substrate (Liberek et al., 1991; Kampinga and Craig, 2010). These events are triggered by the inter-domain communication of the protein (Figure 1.6).

When ATP is bound on the Hsp70\textsubscript{NBD} allosteric communication triggers signals to the Hsp70\textsubscript{SBD} facilitating substrate release (section 1.4.1). Subsequently, hydrolysis of bound ATP causes conformational changes which results in a closed conformation with high affinity for substrate (slow on-off rates) (Figure 1.8; Buchberger et al., 1995; Kiytk et al., 2014). The chaperone cycle of Hsp70 is regulated by two co-chaperones J-domain proteins (Hsp40) (section 1.4.5.1) and
nucleotide exchange factors (NEFs). Of these the most studied co-chaperones are Hsp40 (section 1.4.5) which recruits the substrate and activates ATP hydrolysis on the $Hsp70_{\text{NBD}}$ (Figure 1.8).

1.4.6.4 Nucleotide exchange factors of Hsp70

Nucleotide exchange factors (NEF) improves Hsp70 operational efficiency and acts as substrate release factors (Figure 1.8; Dragovic et al., 2006; Raviol et al., 2006; Sadlish et al., 2008). The NEFs used in prokaryotes and eukaryotes are structurally unrelated but they serve a common role (Raviol et al., 2006). The different types of NEF employed by Hsp70 include; in prokaryotes (and mitochondria); GroP-like gene E (GrpE) (Packschies et al., 1997); in eukaryotes Bcl2-associated athanagene-1 (Bag-1) (Bimston et al., 1998), heat shock protein binding protein 1 (HspBP1) (Patury et al., 2009) and Hsp110 (Dragovic et al., 2006).

NEFs belong to one of the three groups which are the Hsp110/Grp170, HspBP1/Sil1, and Bag domain proteins (Sondermann et al., 2001; Steel et al., 2004, Dragovic et al., 2006). The diversity of NEFs is crucial for functional specialization of Hsp70s as they determine substrate dwell time (Kabani et al., 2008; reviewed in Bracher and Verghese, 2015). The Eubacteria genome encodes for only one Hsp70 NEF which has also been described in organelles such as mitochondria and chloroplasts (Packschies et al., 1997). Eukaryotic genomes encode for several NEFs for example, the human genome encodes for at least thirteen NEFs; four Hsp110, two HspBP/Sil1, five Bag domain proteins and two mitochondrial GrpE (Kampinga et al., 2009).

There are three different nucleotide release mechanisms facilitated by NEF on Hsp70; (i) the action of GrpE, which depends on the formation of β-strand subdomain dimer (Figure 1.9B) which contact subdomains IB and IIB resulting in the rotation of the $Hsp70_{\text{NBD}}$ lobe IIB (Harrison et al., 1997); (ii) in contrast, Hsp110 utilizes the Bag-1 domain which is a three helix bundle where helices 2 and 3 contact subdomains IB and IIB resulting in a 14° rotation of $Hsp70_{\text{NBD}}$ lobe IIB releasing bound ATP (Figure 1.9C) (Sondermann et al., 2001; Andreasson et al., 2008); (iii) HspBP1 embraces $Hsp70_{\text{NBD}}$ lobe IIB and this results in steric conflict which
displaces lobe IB thereby resulting in nucleotide release (Figure 1.9D) (Shomura et al., 2005).

In summary, there are two types of nucleotide releases on Hsp70 which are (i) nucleotide release due to global loss of the tertiary structure of Hsp70\textsubscript{NBD} (HspBP1/Fes1); (ii) nucleotide release through the opening of nucleotide binding cleft of Hsp70\textsubscript{NBD} lobe IIB (Bag-1/GrpE/Hsp110) (Sondermann et al., 2001; Andreasson et al., 2008). The function of NEFs is regulated by a nucleotide ATP promotes NEF function (Buchberger et al., 1994; Andreasson et al., 2008).

![Figure 1.9: The 3D-Model of the human Hsp70 nucleotide binding domain and nucleotide exchange factors](image)

The NBD of hHsp70 (A) showing the channel of nucleotides ADP (purple) movement (arrow) (Wisniewska et al., 2010). The model of nucleotide exchange factors complexed to the Hsp70\textsubscript{NBD} (Red) with the IIB subdomain highlighted (yellow) (B) GrpE - blue and green helices (Harrison et al., 1997), (C) Bag1- green helices (Sondermann et al., 2001) and (D) HspBP1-blue (Shomura et al., 2005).

Genomes of metazoans, plants and some protists harbour a special Hsp70 co-chaperone, Hsp70-interacting protein (Hip) that antagonizes NEFs function on Hsp70 (Connell et al., 2001). Hip binds to the Hsp70\textsubscript{NBD} forming a bracket that locks the Hsp70\textsubscript{NBD}, preventing the release of ADP from the nucleotide binding cleft (Li et al., 2013). Hip regulates the release of substrates that are to be passed over to downstream chaperones like Hsp60 (section 1.4.4)
or Hsp90 (sections 1.4.4; Li et al., 2013). Hence Hip is also involved in the suppression of aggregation, in that it allows substrates whose reactive patches are exposed to remain locked in the Hsp70\textsubscript{SBD} until the environment is conducive for their refolding enabling selective substrate release from Hsp70 complex (Li et al., 2013).

### 1.4.6.5 *P. falciparum* Hsp70s

The *P. falciparum* genome encodes for six Hsp70s which are localized in specific cellular compartments (Table 1.2; Shonhai et al., 2007). The different localization profiles of Hsp70s help to serve for role specialization. Based on bioinformatics data and yeast two hybrid assays, *P. falciparum* Hsp70s are thought to interact with proteins in virtually every stage of its life cycle, facilitating protein folding, refolding and for degradation (Table 1.2; Shonhai et al., 2007; Przyborski et al., 2015).

**Table 1.2: PfHsp70 and their characteristics**

<table>
<thead>
<tr>
<th>Features</th>
<th>Molecular mass</th>
<th>Chromosome</th>
<th>Location</th>
<th>Signal sequence</th>
<th>Expression phase (mRNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PfHsp70-1\textsuperscript{a} (PF3D7_0818900)</td>
<td>74</td>
<td>8</td>
<td>nucleus and cytosol\textsuperscript{b}</td>
<td>C terminal EEVD motif\textsuperscript{a}</td>
<td>exo-erythrocytic stage\textsuperscript{a}</td>
</tr>
<tr>
<td>PfHsp70-2\textsuperscript{bc} (PF3D7_0917900)</td>
<td>73</td>
<td>9</td>
<td>ER\textsuperscript{b}</td>
<td>carboxylic ER sequence\textsuperscript{d}</td>
<td>exo-erythrocytic stage\textsuperscript{c}</td>
</tr>
<tr>
<td>PfHsp70-3\textsuperscript{a} (PF3D7_1134000)</td>
<td>73</td>
<td>11</td>
<td>mitochondrial\textsuperscript{ad} NC</td>
<td>mitochondrial transit sequence\textsuperscript{de}</td>
<td>NE</td>
</tr>
<tr>
<td>PfHsp70-x (PF3D7_0831700)</td>
<td>76</td>
<td>7</td>
<td>Rbc cytosol, J-dots\textsuperscript{f}</td>
<td>C terminal EEVN motif iRbc export signal motif ASNNAEES; ER signal sequence MKTKICSYYHVFLISTTVHT\textsuperscript{f}</td>
<td>intra-erythrocytic stage\textsuperscript{f}</td>
</tr>
<tr>
<td>PfHsp70-y (PF3D7_1344200)</td>
<td>108</td>
<td>13</td>
<td>ER\textsuperscript{d}</td>
<td>carboxylic ER sequence\textsuperscript{d}</td>
<td>ER</td>
</tr>
<tr>
<td>PfHsp70-z (PF3D7_0708800)</td>
<td>100</td>
<td>7</td>
<td>cytosol\textsuperscript{d}</td>
<td>NE</td>
<td>NE</td>
</tr>
</tbody>
</table>

ER (Endoplasmic Reticulum), , NE (Not Established), PlasmoDB ascension numbers are in brackets next to protein, the superscript letters represent the following references: a- Sharma et al (1992); b- Kumar et al (1991); c- Nyalwidhe and Lingelbach (2006); d- Sargent et al (2006); e- Sato and Wilson (2003); f- Külzer et al (2012); g- Muralidharan et al (2012), adapted from Shonhai et al (2007).
1.4.6.5.1 *P. falciparum* Hsp70-1

One of the most prominent chaperones PfHsp70-1, localises mainly in the cytosol and nucleus (Table 1.2; Shonhai *et al*., 2007). PfHsp70-1 is stress inducible and is expressed at the blood stages of the parasite (Sharma, 1992; Kappes *et al*., 1993). PfHsp70-1 is known to function as a chaperone responsible for thermotolerance (Shonhai *et al*., 2005; Bell *et al*., 2011). *In vitro* studies using the recombinant form of PfHsp70-1, it was shown that the protein suppresses heat induced aggregation of model substrate malate dehydrogenase (Shonhai *et al*., 2008). Stephens and colleagues (2011), showed that PfHsp70-1 is required for folding of heterologous expressed recombinant *P. falciparum* GTP cyclohydrolase. This suggests that PfHsp70-1 facilitates protein folding, which defines its chaperone cytoprotective role in parasite survival during febrile episodes.

1.4.6.5.2 *P. falciparum* Hsp70-2

PfHsp70-2/*P. falciparum* immunoglobulin heavy chain binding protein (PfBiP) is stress inducible and is expressed during the blood stages of the parasite life cycle (Table 1.2) (Kumar *et al*., 1991; Sharma, 1992; Sommer *et al*., 2007). PfHsp70-2 protein shares 53 % sequence identity with PfHsp70-1 and as such it is the ER resident member of PfHsp70s (Shonhai *et al*., 2007; Hempel *et al*., 2009). PfHsp70-2 facilitates the import of proteins into the ER and to ensure they are folded properly. The importation of proteins into the ER is thought to be through the prominent translocon composed of *P. falciparum* Sec (PfSec) complex (Sommer *et al*., 2007; Reviewed in Shonhai, 2014). PfHsp70-2 binds to and detaches from the translocon on the ER lumen thus allowing extended proteins to pass through the PfSec complex translocon in a nucleotide dependent fashion (Sommer *et al*., 2007). In the ADP bound state, PfHsp70-2 binds to the translocon preventing compact proteins to enter the ER lumen and in the ATP bound state it detaches from the translocon allowing entry of extended protein into the ER lumen (Saridaki *et al*., 2008). Once it opens up the translocon it binds to the extended peptide and it is thought that it pulls the peptide into the ER thus avoiding it sliding back into the cytoplasm (reviewed in Shonhai, 2014). In the ER the protein quality control is thought to be centrally controlled by PfHsp70-2 which is responsible for maintaining
proteins in native functional state. The proteins which are damaged are thought to be channeled for degradation through the ER-associated degradation (ERAD) pathway.

1.4.6.5.3 P. falciparum Hsp70-3

PfHsp70-3 is localized in the matrix side of the mitochondrion (Table 1.2) (Sato and Wilson et al., 2004), and has been shown to interact with asparagine rich proteins (La Count et al., 2005). The mitochondrion genome encodes for only two functional proteins, this suggests that most of the proteins that function in the matrix are nuclear encoded (Gardner et al., 2002). These proteins are translocated through the two mitochondrion membranes with the assistance of PfHsp70-1 on the cytosol side and facilitated by PfHsp70-3 on the mitochondrion matrix (Figure 1.10).

![Figure 1.10: The role of PfHsp70-3 during import of proteins into mitochondria](image)

Preproteins coming from the translational machinery are bound by PfHsp70-1 and allowed to thread into the TOM complex through the assistance from the receptors on the outer membrane complex. The preproteins then translocase the intermembrane space and thread through the Tim complex where they are sequestered with the assistance of PfHsp70-3 and PfPam18. The activity of PfHsp70-3 is modulated by GrpE and Hsp40s resident in the mitochondria. The preproteins are refolded by the Hsp60/hsp10 complex, adapted from Shonhai (2014).
To traverse these two membranes peptides are first extended and kept in folding competent conformation by PfHsp70-1 then passed through the Translocase of the outer membrane complex (Tom) (Njunge et al., 2013) and they thread through the inter-membrane space into the translocase of the inner membrane (Tim23) complex (Pfanner and Wiedemann, 2002), possibly by the pulling from PfHsp70-3. GrpE and Hsp40 co-chaperones in the mitochondrion modulate the function of PfHsp70-3 (Figure 1.10). The pre-proteins are then folded to functional proteins by the PfHsp60/10 chaperonin system (Sato and Wilson, 2004).

1.4.6.5.4 P. falciparum Hsp70-x

PfHsp70-x is a close homologue of PfHsp70-1 that localizes in the PV and is exported to the iRBC cytosol (Table 1.2; Külzer et al., 2012). PfHsp70-x was shown to be expressed and exported during all the developmental stages of the parasite blood stage (Figure 1.11; Külzer et al., 2012). The export of parasite proteins to the host RBC cytosol is thought to be mediated by the presence of an export signal of the protein sequence (PEXEL/VTS; Hiller et al., 2004; Marti et al., 2004). Interestingly, PfHsp70-x lacks the PEXEL/VTS signal. This suggests that its export to the iRBC cytosol is mediated through another signal mechanism. The Przyborsky group proposed that the first 25 amino acids on the N-terminus of PfHsp70-x represent a form of an ER signal sequence (MKTKICSYIHIVLFLISTTVHT), followed by 8 residues that come after the iRBC export signal motif (ASNNAEES; Külzer et al., 2012). This suggests that there is post translational modification to PfHsp70-x where the N-terminus MKTKICSYIHIVLFLISTTVHT signal sequence targets PfHsp70-x translocation by the classical ER targeting. PfHsp70-x is thought to be packaged into vesicles that will fuse with parasite membrane moving into the PV. When PfHsp70-x is in the PV, it is thought that the signal sequence ASNNAEES targets the protein to the iRBCs cytosol (Figure 1.11; Külzer et al., 2012).
Hsp70s are functionally regulated by Hsp40s (section 1.4.5.1; Kampinga and Craig, 2010). Several reports show evidence that parasite encoded DnaJ/Hsp40 proteins are exported to the iRBC cytosol including PfEMP1 (sections 1.2.4; Hiller et al., 2004; Maier et al., 2008; Kültzer et al., 2010). It was shown that PfHsp70-x co-localizes with type II Hsp40s (PF3D7_0113700 and PF3D7_0501100.1) in structures termed ‘J-dots’ (Kützer et al., 2010). J dots are mobile parasitic derived structures present in the iRBC cytosol (Kützer et al., 2010). This suggests the presence of Hsp70-Hsp40 functional complexes in the iRBC cytosol (Kützer et al., 2012). The deployment of parasite proteins to the iRBC membrane is thought to be facilitated by parasite heat shock proteins. For example, PfHsp70-x is thought to facilitate the deployment of PfEMP1 (Wickham et al., 2001). This process may be facilitated through the co-operative function of the Hsp70-Hsp40 complex. PfHsp70-x is thought to be the chaperone that is central to the folding of exported parasite proteins (Kützer et al., 2010).

The presence of PfHsp70-x in the PV suggests that it may function as a chaperone which maintains the extended peptides folding competent form in the PV unfolded (Kützer et al., 2010; Grüring et al., 2012). The PfClp (section 1.4.2) is part of the Plasmodium translocon of...
exported proteins (PTEX) on the PVM (Figure 1.10; de Koning-Ward et al., 2009). As shown in other species that Hsp70 and Clp (section 1.4.2) interact in their role as disaggregase it is not clear if in *P. falciparum* PVM context they interact in assisting the translocation of proteins through the PVM. Banumathy and colleagues, (2003) have demonstrated the presence of human Hsp90 and human Hop in iRBCs. The absence PfHop in the iRBC suggest that the EEVN motif of PfHsp70-x may be to facilitate its interaction with human Hop and Hsp90. This suggests that PfHsp70-x interacts with human Hsp90 and Hop in facilitating proper folding of parasite driven proteins exported to the RBC cytosol.

**1.4.6.5.5 Hsp110 family of proteins**

Heat shock protein 110 (Hsp110) is a member of the Hsp70 super-family of molecular chaperones (Table 1.1; Oh et al., 1999; Raviol et al., 2005). Hsp110s have been identified in the cytosol while Grp170 occurs in ER (Oh et al., 1999; Saxena et al., 2012; Xu et al., 2012). Like canonical Hsp70s, the structure of Hsp110 comprises of N-terminal nucleotide binding domain (Hsp110\_NBD), followed by substrate binding domain (Hsp110\_SBD) (Figure 1.6). Hsp110s have larger substrate binding domains and have significant sequence divergence from the archetypical Hsp70 (Oh et al., 1999). The Hsp110\_SBD is divided into three sub-domains; the β sandwich which is responsible for peptide binding (Domain B), followed by long acidic loop subdomain (Domain L), and then the three helix sub-domain (Domain H) at the C-terminal domain which function as the cap for large substrate holding capability also called the ‘lid’ (Figure 1.12; Oh et al., 1999; Polier et al., 2010). Hsp110 proteins possess a fairly conserved NBD (Domain A; Figure 1.12). However, Hsp110 linker segment and Hsp110\_SBD are divergent from those of the canonical Hsp70s (Polier et al., 2010). Hsp110s are reported to lack allosteric coupling between the Hsp110\_NBD and Hsp110\_SBD (Mayer, 2010). Consequently, Hsp110s have been found to exhibit chaperone function limited to holdase function (protein aggregation suppression; Goeckeler et al., 2002; Polier et al., 2010).

Hsp110s exhibit similar domain architecture as Hsp70 with an extra insertion on SBD\_β and longer C-terminal loop both of which are thought to be dispensable for holdase function
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(Shaner et al., 2004; Liu and Hendrickson, 2007). Hsp110 lack refolding activity but they have been described as exhibiting more efficient holdase function than canonical Hsp70s (Oh et al., 1997; Goeckeler et al., 2002). Hsp110s’ holdase activity has been suggested to be responsible for their contribution in prion formation and propagation (Sadlish et al., 2008), ER degradation (Hrizo et al., 2007) and tumour antigen presentation (Manjili et al., 2002; Wang et al., 2001).

In humans and mice three distinct cytosolic Hsp110 homologs exist; Hsp105 (splice variants α and β); Apg-1 and Apg-2 (Easton et al., 2000; Kampinga et al., 2009). Hsp105 variants are similar to S. cerevisiae Hsp110 isoforms where Hsp105α is constitutively expressed and Hsp105β is expressed under heat stress (Easton et al., 2000; Hatayama et al., 2001). On the other hand, Apg1 and Apg-2 are developmentally expressed in various tissues (Kaneko et al., 1997a; Kaneko et al., 1997b). Yeasts express two cytosolic Hsp110/Sse isoforms (Sse1p and Sse2p) which are functional under different conditions. Sse1p has been shown to function both as a NEF and a holdase at normal growth temperature ~ 37 °C (mild heat stress; Polier et al., 2008). Interestingly, at elevated heat stress, Sse1p has reduced NEF functionality as
compared to its isoform Sse2p (Polier et al., 2008). This suggests that, these isoforms may functionally complement each other at different temperatures.

**1.4.6.5.1 Hsp110 substrate binding**

The crystal structure of the Hsp110 is complex with substrates unravelled that both the Hsp110 NBD and the SBD β-sandwich are required for substrate binding (Polier et al., 2010). The Sse1p crystal structure showed the presence of a rift between the NBD and SBD interfaces. This rift is thought to grasp substrates; consequently, hiding the hydrophobic patches of misfolded proteins (Polier et al., 2010). The mechanism of binding is similar to the binding of trigger factor (a ribosome associated prolyl isomerase of prokaryotes) (Hesterkamp et al., 1996; Hoffman et al., 2010), prefoldin (a hexameric molecular chaperone complex; Seigert et al., 2000) and Skp (a molecular chaperone of the periplasmic space of E. coli; Schäfer et al., 1999).

The second mechanism proposed to be used for substrate binding by Hsp110 is partial thermal unfolding of Hsp110 through the creation of an intermediate conformation under heat stress with high affinity for substrate binding (Haslbeck et al., 2005; Polier et al., 2010). In this conformation the hydrophobic motifs of N- and C-terminal extensions of Hsp110 are able to efficiently bind to hydrophobic patches on partially unfolded substrates (Haslebeck et al., 2005). Thus, Hsp110 keeps the substrates in a folding competent state during heat stress (Polier et al., 2010). This possibly explains the ability of Hsp110 to hold larger substrates (Polier et al., 2010). In support of this mechanism of action, Sse1p was embedded in the three dimensional meshwork of aggregated luciferase, and thus shielding luciferase molecules from each other, hence reducing aggregation (Polier et al., 2010). When the thermal stress was removed, luciferase was made available to Hsp70 for refolding (Raviol et al., 2006; Polier et al., 2010).
1.4.6.5.2 Hsp110 functional cooperation with Hsp70

Shorter (2011) reported that in the absence of Hsp100, the mammalian cytosol harbours an ATP-depended disaggregase complex composed of Hsp110, Hsp70 and Hsp40. This functional complex was shown to slowly dissolve large disordered aggregates and recover natively folded protein from two model proteins; urea denatured luciferase that form aggregated structures of approximately 500 - 2000 kDa (Glover and Lindquist, 1998) and heat denatured Green Fluorescence Protein (GFP) which forms continuous aggregated sequences of approximately 500 kDa (Doyle et al., 2007). The co-operation of Hsp70, Hsp110 and Hsp40 in entangling aggregated proteins required ATP to function (Shorter, 2011).

Hsp110s regulates yeast prion formation and propagation (Sadlish et al., 2008). Yeast productively utilises prion as epigenetic translation regulators which in other systems are cytotoxic and lead to cell death (Sondermann et al., 2002). Some prions are used as signals for translation termination in regulating gene expression (Sadlish et al., 2008). The most studied prion is PSI+, the altered form of the translational release factor Sup35 (Sadlish et al., 2008). Several studies have demonstrated that Hsp110 is essential for efficient de-novo PSI+ prion formation and PSI+ phenotype propagation (Fan et al., 2007; Kryndushkin and Wickner, 2007; Sadlish et al., 2008). Here Hsp110 possibly functions both as a NEF for Hsp70 (Goeckeler et al., 2002) and as a holdase stabilising folding intermediates in an active form for prion Sup35 propagation (Yam et al., 2005; Sadlish et al., 2008).

1.4.6.5.3 P. falciparum Hsp70-y

PfHsp70-y (Table 1.2) belongs to the Hsp110 group and it is the ER localized member Grp170 (Shonhai et al., 2007). PfHsp70-y is likely to serve as a NEF for the ER localized PfHsp70-2 (Shonhai, 2014). In the ER, there is a high rate of generation of hydrogen peroxide through the degradation of hemoglobin coupled with the action of antimalarial drugs that induce oxidative stress (Akide-Ndinge et al., 2009; Hartwig et al., 2009). For survival the parasite needs a robust protein quality control system.
1.4.6.5.4 *P. falciparum* Hsp70-z

PfHsp70-z (Table 1.2) belongs to the Hsp70 superfamily but is phylogenetically a close member of Hsp110 protein family (Shonhai *et al*., 2007). The Hsp110 protein family members are thought to exhibit NEF activity on Hsp70 (Dragovic *et al*., 2006). PfHsp70-z has not been biochemically characterised but is thought to function as a chaperone (Muralindaran *et al*., 2012). It is predicted to interact with PfHsp70-1 (section 1.4.6.5.1), PfHsp90 (section 1.4.3; La Count *et al*., 2005) and is also proposed to serve as a NEF (Shonhai *et al*., 2007; Bhartiya *et al*., 2015). Hsps are essential for the survival of *P. falciparum* parasite in both othrotherm and homoeothermic hosts. *P. falciparum* proteome is thought to comprise of at least 25% of proteins with asparagine repeats (Singh *et al*., 2004b). These repeats increase the propensity of these proteins to aggregate under heat stress. It is thought that PfHsp70-z prevents the aggregation of asparagine rich parasitic proteins under stressful conditions (Muralindaran *et al*., 2012). Some heat shock proteins have been shown to be essential in parasite development. PfHsp90 (section 1.4.3) is essential for the development of the parasite (Banumathy *et al*., 2003; Shonhai *et al*., 2007). Further elucidation of the role of PfHsp70-z in parasite survival will enhance the understanding of its role in protein folding and as a potential drug target.

1.5 Problem Statement, Hypothesis, Aim and Objectives

1.5.1 Problem statement

*P. falciparum* Hsp70-1 (PfHsp70-1; section 1.4.6.5.1), which primarily localizes to the cytosol and nucleus is thought to confer cytoprotection to the parasite (Shonhai *et al*., 2007). The regulation of PfHsp70-1 activity by Hsp40 co-chaperones has been presented as a prospective pathway for drug design (Shonhai, 2010; Njunge *et al*., 2013). However, little is known about how PfHsp70-1 is regulated by NEFs. Nucleotide exchange defines the life-span of the interaction of Hsp70 with its substrate and has an influence on whether the substrates are refolded or channelled towards degradation. The successful design of inhibitors targeting the function of Hsp70 in *P. falciparum* (Shonhai *et al*., 2010), requires knowledge on how the nucleotide exchange function Hsp70 is regulated. NEFs found in the cytosol of eukaryotic cells include, homologues of BAG domain proteins; HspBP1, and Hsp110 families (Maier *et al*.,
Although PfHsp70-1 possesses putative binding motifs for NEFs based on bioinformatics analysis (Shonhai et al., 2007), BAG1 and HspBP1 homologues have not been identified in *P. falciparum*. However, *P. falciparum* Hsp70-z (PfHsp70-z) [cg4] (section 1.4.6.5.5.4), a member of the Hsp110 family was reportedly expressed by parasites isolated from clinical cases (Pallavi et al., 2010). This suggests that PfHsp70-z is the sole NEF for cytosolic PfHsp70-1.

The PfHsp70-z encoding gene occurs on chromosome 7 (Shonhai et al., 2007) which houses antigenic var and drug-resistant pfcr genes. Hence, PfHsp70-z may play a role in parasite virulence and drug resistance. The function of cytosolic Hsp110 is essential in yeast (Moran et al., 2013). Human and yeast Hsp110 proteins exhibit variable functional features (Polier et al., 2010). Thus Hsp110 proteins may present species specific functional features appropriate for possible drug design.

1.5.2 Hypothesis

PfHsp70-z is an independent chaperone that interacts with PfHsp70-1 to facilitate the nucleotide exchange function of the latter.

1.5.3 The main aim of this study

To biochemically characterise the role of *P. falciparum* heat shock protein 70-z.

1.5.4 Objectives

1.5.4.1 To utilise bioinformatics analysis to identify the structure-function features of PfHsp70-z.

*Approach*
a) Retrieval of PfHsp70-z sequence from plasmoDB, and BLAST searches to identify homologues;

b) Sequence alignments with retrieved homologues to identify conserved functional domains using BioEdit/MAFFT;

c) Generation of three dimensional model of the protein to predict possible interaction sites with PfHsp70-1; and

d) Predict possible PfHsp70-z interactors using STRING.

1.5.4.2 To investigate chaperone activity of PfHsp70-z and its interaction with nucleotides using biochemical analysis.

Approach

a) Heterologous expression and purification of recombinant PfHsp70-z protein from E. coli;

b) Investigate nucleotide binding kinetics using surface plasmon resonance (SPR) and ATPase activity of recombinant PfHsp70-z using colorimetric assay;

c) Investigate the effect of nucleotides (ATP/ADP) on PfHsp70-z structure using limited proteolysis and tryptophan fluorescence;

d) Investigate the capability of PfHsp70-z to suppress heat induced aggregation of model substrates luciferase and malate dehydrogenase; and

e) Investigate the interaction of recombinant PfHsp70-z and PfHsp70-1 using slot blot and SPR analyses.

1.5.4.3 To investigate the localisation of PfHsp70-z and PfHsp70-1 in parasite infected erythrocyte.

Approach

a) Investigate the localisation of PfHsp70-z in parasite infected erythrocytes using immunofluorescence microscopy.
1.5.4.4 To investigate the expression of PfHsp70-z in parasites subjected to heat stress.

*Approach*

a) Investigate the expression levels of PfHsp70-z in *P. falciparum* 3D7 infected cells grown at 37 °C and heat shocked at 42 °C; and

b) Investigate the stability of recombinant PfHsp70-z to heat stress using circular dichroism (CD).

1.5.4.5 To explore the association of PfHsp70-z and PfHsp70-1 using co-immunoprecipitation assays.

*Approach*

a) Immuno-precipitation assays will be conducted using α-PfHsp70-z antibodies.
CHAPTER 2:

BIOINFORMATICS ANALYSES OF THE STRUCTURAL FEATURES OF PFHSP70-Z


Chapter 2:
Bioinformatics Analyses of the Structural Features of PfHsp70-z

2.1 Introduction

Bioinformatics has been at the forefront in the analysis of DNA and protein sequences. It has been useful in the prediction of the secondary structure and the three dimensional structure of protein. Bioinformatics is also employed to predict protein-protein interactions. Protein-protein interactions reveal the possible role of unknown proteins through their interaction with functional partners. Due to the growth in genomic information mining, several databases have been created which are dedicated to housing species specific genomic data. For example, PlasmoDB (http://plasmodb.org/plasmo/; Aurrecoechea et al., 2009) contains extensive genome, proteome and metabolome information relating to malaria parasites (Table 2.1). PlasmoDB is an interactive database that allows inter and intra species genome comparisons (Otto et al., 2014). The P. falciparum strains represented in online databanks include HB3, DD2, D10, 7G8 and 3D7 (Mu et al., 2002). PlasmoDB houses P. falciparum genome sequence and annotation as well as draft sequences emerging from several Plasmodium sequencing projects. PlasmoDB also houses eight plasmodium parasite species with full sequences and annotated genomes (Table 2.1).

P. falciparum genome sequencing was started in 1996 and completed in 2002 and was recently updated (Gardner et al., 2002; Otto et al., 2014). The genome consists of 14 chromosomes distributed into three compartments, the nucleus, mitochondrion and the apicoplast, with 8 % being nuclear genome. The genome has high AT composition, with 54 % composed of introns which results in about 2.3 kb of gene length. The nucleus genome codes for both tRNA and ligases. The mitochondrial genome comprises of 5.8 kb and has limited protein coding genes (Gardner et al., 2002). This suggests that most of the proteins functioning in the mitochondria are imported from the parasite cytosol.

In silico analysis revealed that of the ~5300 protein genes, the P. falciparum genome appears to code for approximately 95 different chaperones (Pavithra et al., 2007). At least six Hsp70
genes have been identified in *P. falciparum*, amongst these, two belong to the Hsp110 family PfHsp70-z (section 1.4.6.5.4) and PfHsp70-y (section 1.4.6.5.3) (Table 1.2; Shonhai *et al.*, 2008). An *Hsp110* homologue (*PfHsp70-z*), has been identified on the genome (Shonhai *et al.*, 2007; Muralidharan *et al.*, 2012). Identification of the residues that are implicated in PfHsp70-z nucleotide exchange and chaperoning function will help elucidate the role of PfHsp70-z in parasite survival. Hsp110 are known to function as NEFs for canonical Hsp70s (Dragovic *et al.*, 2006). There is a possibility that PfHsp70-z may function as both NEF and as a chaperone.

The main aim of this study was to utilise bioinformatics analyses to identify structural and functional motifs and domains in PfHsp70-z. The structural features of PfHsp70-z will be compared to those of its homologues. The specific objectives of this study were to:

i. Identify and compare the cytoplasmic PfHsp70-z protein homologues in *Plasmodium spp*, *Trypanosomes*, human, yeast and mouse;

ii. Map out the phylogeny of PfHsp70-z and putative homologues;

iii. Generate the three dimensional model of PfHsp70-z;

iv. Identify a unique peptide on PfHsp70-z against which a specific antibody was generated;

v. Assess the genomic neighbourhood of PfHsp70-z by chromosomal gene locus analysis; and

vi. Predict and construct the predicted interactome of PfHsp70-z.
2.2 Experimental Procedures

2.2.1 Multiple sequence alignment of Hsp110 homologues

The amino acid sequence representing PfHsp70-z retrieved from PlasmoDB (Accession number: PF3D7_0708800) was aligned to sequences of other cytosolic Hsp110 homologues from the following: *Plasmodium* species retrieved from PlasmoDB in FASTA format (www.PlasmoDB.org); Tritryps Hsp110 retrieved from TritrypDB in FASTA format (http://tritrypdb.org); *Homo sapiens* and *Mus musculus* Hsp110 sequences were retrieved in FASTA format from National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov). The retrieved protein sequences and their respective accession number are as follows; *P. falciparum*, PfHsp70-z (PF3D7_0708800); *P. vivax*, PvHsp110 (PVX_087970); *P. knowlesi*, PkHsp110 (PKH_010690); *P. chabaudi*, PcHsp110 (XP_745506.1); *P. berghei*, PbHsp110 (PBANKA_121930); *P. yoelii*, PyHsp110 (PlasmoDB accession number PYYM_1222000); *P. cynamolgi*, PcyHsp110 (PCYB_011590); *Trypanosoma brucei*, TbHsp110 (TB927.10.12710); *T. cruzi*, TcHsp110 (TcCLB.507831.60); *Leishmania major*, LmHsp110 (LmjF.18.1370); *L. braziliensis*, LbHsp110 (LbrM.18.1400); *Homo sapiens*, HSPH1 (NP_006635.2); *Mus musculus*, mHsp110 (NP_038587) and *Saccharomyces cerevisiae*, Sse1 (Q875V0). The retrieved protein sequences were analysed using MAFFT version 7 (Katoh et al., 2013) and Boxshade (http://www.ch.embnet.org/software/BOX_form.html).

2.2.2 PfHsp70-z homology modelling

The PfHsp70-z sequence retrieved from PlasmoDB was used to conduct a BLAST search to identify the best possible template sequence for homology modeling. Sse1 sequence was identified as it shared the highest possible sequence identity with PfHsp70-z. Template sequence of Sse1 and crystal structure were used to construct three dimensional models of the PfHsp70-z nucleotide binding domain and substrate binding domains using PHYRE2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index; Kelley et al., 2015). The images were visualized using Chimera version 1.9 (Pettersen et al., 2004). The resulting PDB files were used for Chimera match making PfHsp70-z onto Sse1.
2.2.3 Secondary structure prediction of PfHsp70-z

The prediction of the secondary structure of PfHsp70-z C-terminal was achieved by identifying a template sequence from multiple sequence alignment conducted using amino acid sequences derived from PlasmoDB. The template sequence and crystal structure were analysed and used to predict the secondary structure using PHyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index; Kelly et al., 2015).

2.2.4 Design of peptide for anti-peptide PfHsp70-z antibody

To design a peptide to be used to generate the antibody for PfHsp70-z, the DNASTAR (http://www.dnastar.com) composite analysis was used. The analysis was conducted to determine antigenic regions that are surface exposed and unique to PfHsp70-z using DNASTAR (Proteone, Proteon3D and Novafold) plugins. The epitope determination analysis included assessment of antigenicity using Jameson-Wolf Index plots (Jameson and Wolf, 1988); secondary structure prediction using Chou-Fasman plots (Chou and Fasman, 1978); charge density analysis using Lehninger plots (Lehninger, 2005); chain flexibility using the Karplus-Schultz plots (Karplus et al., 1978) and hydrophobicity and hydrophilicity using Kyte-Doolittle plots (Kyte and Doolittle, 1982). To determine the overall antigenicity the Jameson-Wolf Antigenic index which combines data from existing protein structural predictions was used (Jameson and Wolf, 1988). An antigenic region with the lowest similarity to any other Plasmodium protein was assessed using BLAST and the region with high antigenicity and lowest similarities to human and other Plasmodium homologues was selected. The peptide sequence (CSLQEQEKNKPLYEP) corresponding to amino acids 768–782 of the PfHsp70-z amino acid sequence was selected and submitted to the GenScript corporation (Piscataway, New Jersey, USA) to be synthesized. Once synthesized, GenScript used the peptide to produce polyclonal antibody against PfHsp70-z in a rabbit.

2.2.5 PfHsp70-z genome neighbourhood analysis

Genes in close proximity are more likely to undergo similar epigenetic modifications, interactions and transcriptional activity (Overbeek et al., 1999; De and Babu, 2010). PfHsp70-
z gene location was assessed to determine important gene neighbours using data retrieved from PlasmoDB (Aurrecoechea et al., 2008).

2.2.6 Mapping out the predicted interactome of PfHsp70-z
The interactome prediction for PfHsp70-z was conducted using data downloaded from STRING 10 (http://string-db.org/) database. STRING works as a meta-database which integrates data collected from several sources including gene neighbourhood, co-expression experiments, databases and text mining (Jensen et al., 2009). The network was constructed displaying nodes and edges with a median confidence of 0.7. The nodes and edges were analysed on Cytoscape (Shannon et al., 2004). A NetworkAnalyzer tool (Brandes, 2001) was used to establish betweenness centrality and closeness centrality. The node size was mapped to the betweenness centrality of PfHsp70-z to reflect the level of control it exerts over the interactions of other proteins in the network (Yoon et al., 2006). The node colours were mapped to closeness centrality as a measure of the speed information spreads from PfHsp70-z to other reachable nodes in the network (Newman, 2003).
Chapter 2:
Bioinformatics Analyses of the Structural Features of PfHsp70-z

2.3 Results

2.3.1 Analysis of sequence similarity between PfHsp70-z, Plasmodium, Trypanosoma, Leishmania, human, yeast and mouse homologues

Multiple sequence alignments were conducted on the predicted cytosolic full length Hsp110 proteins from Plasmodium species and Trityps (T. cruzi, T. brucei, L. major, and L. brasilienz) were compared to human, mouse and yeast orthologues (Figure 2.1; 2.2). PfHsp70-z possesses an N-terminal NBD (PfHsp70-zNBD) composed of residues 1 - 420 (Figure 2.1), a putative linker region at position 421 - 426 (Figure 2.1) and a SBD composed of SBDβ residues 422 - 660 and SBDα residues 661 - 729 and a C-terminal loop, residues 730 - 873 (Figure 2.2) (Oh et al., 1999). Hsp110 residues that are known to be important for the interaction with Hsp70 (Figure 2.1) (Schuermann et al., 2008), are more highly conserved in the PfHsp70-zNBD compared to those located in PfHsp70-zSBD (Figure 2.1; 2.2).

The proposed linker residues (EYECVE) (section 1.4.6) of Hsp110s from Plasmodia species are highly divergent from those of yeast Hsp110 (PFKFED) (Schuermann et al., 2008) as well as human Hsp110 and mouse Hsp110 (EFSVTD) (Figure 2.1). On the other hand the EFVVSE representing the linker of Hsp110 from Trypanosomes and Leishmania is also divergent from yeast Hsp110s (Figure 2.1). The divergent linker sequences may have evolved due to species specific functional needs. These findings are in line with previous findings that yeast and human Hsp110 show functional differences (Oh et al., 1999; Goeckeler et al., 2008; Polier et al., 2008).

The residues that are implicated in the formation of homodimers and heterodimers are conserved in PfHsp70-z (Figure 1.2). This suggests that PfHsp70-z possibly form functional homodimers and form heterodimers with PfHsp70-1. These finding are in agreement with evidence from previous studies that suggest that Hsp110 form functional heterodimers with Hsp70s (Liu and Hendrickson, 2007; Malinverni et al., 2015).
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ATP-bound Hsp110s demonstrate stable interactions with Hsp70s (Andresson et al., 2008).

The residues required for ATP binding on the Hsp110NBD are highly conserved; 94.1% among plasmodial Hsp110 and 72.5% across all Hsp110 homologues (Figure 2.1). This suggests that nucleotide binding is an important characteristic based on the level of conservation and thus PfHsp70-z possibly interacts with PfHsp70-1 and ATP promotes the interaction. Furthermore,

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**Figure 2.1 Multiple sequence alignment of NBD from Hsp110 homologues**

Multiple sequence alignment of Hsp110NBD homologues. Protein sequence accession numbers were obtained from PlasmoDB (Aurrecoechea et al. 2008); TritrpDB (Aslett et al., 2010) and NCBI (Geer et al. 2010). P. falciparum, PfHsp70-z (PlasmoDB accession # PF3D7_0708800); P. vivax, PVXHsp110 (PlasmoDB accession # PVX_087970); P. knowlesi, PKKHsp110 (PlasmoDB accession # PKH_010690); P. chabaudi, PCHHsp110 (PlasmoDB accession # XP_745506.1); P. berghei, PBAHsp110 (ANKA, PlasmoDB accession # PBANKA_121930); P. yoelli, PYYHsp110 (PlasmoDB accession # PYYM_1222000); P. cynomogoli, PCYHsp110 (PlasmoDB accession # PCB_011590); Homo sapiens, HSPH1 (NCBI accession # NP_006635.2); Mus musculus, mHsp110 (NCBI accession # NP_083857); S. cerevisae, Sse1 (NCBI accession # Q875V0); T. brucei, TbHsp110 (accession # Tb927.12710); T. cruzi, TcHsp110 (accession # TcCLB.2057831.60); L. major, LmHsp110 (accession # Lmjf.18.1370) and L. braziliense, LbHsp110 (accession # LbrM.18.1400). Identical residues are presented in white against a black background, whilst similar residues are shown in white against a grey background the shading was done using Boxshade programme (http://sourceforge.net/projects/boxshade/) ClustalW based alignment was done using MAFFT online tool (http://mafft.cbrc.jp/alignment/software/) to carry out the multiple sequence alignment. The following references represented by superscript (a) Shonhail et al., (2008); (b) Liu and Hendrickson, (2007); (c) Schuermann et al., (2008); (d) Moran et al., (2013); (e) Wisnieska et al., (2010); (f) Kityk et al., (2012).
the Hsp110 residues implicated in protein reorientation after ATP binding (Figure 2.1) (Moran et al., 2013), are well conserved in PfHsp70-z. This suggests that PfHsp70-z possibly exhibits conformational changes in response to nucleotide binding which may be important for regulation its functional cycle.

<table>
<thead>
<tr>
<th>Protein</th>
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<tr>
<td>PfHsp70-z</td>
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<tr>
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| PBAHsp110   | 461     |
| PYYHsp110   | 461     |
| PCHHsp110   | 461     |
| PKHHsp110   | 458     |
| PVXHsp110   | 458     |
| PCYHsp110   | 447     |
| TbHsp110    | 444     |
| TcHsp110    | 444     |
| LmHsp110    | 444     |
| LaHsp110    | 444     |
| LbHsp110    | 444     |
| HSPH1       | 428     |
| mHsp110     | 428     |
| Ssel        | 427     |

| PfHsp70-z   | 513     |
| PBAHsp110   | 515     |
| PYYHsp110   | 515     |
| PCHHsp110   | 512     |
| PKHHsp110   | 512     |
| PVXHsp110   | 512     |
| PCYHsp110   | 510     |
| TbHsp110    | 502     |
| TcHsp110    | 502     |
| LmHsp110    | 503     |
| LaHsp110    | 503     |
| LbHsp110    | 503     |
| HSPH1       | 483     |
| mHsp110     | 483     |
| Ssel        | 483     |

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Figure 2.2: Multiple sequence alignment of SBD of Hsp110 homologues

Multiple sequence alignment of Hsp110 SBD orthologs and homologues. The accession numbers are as described in figure 2.1. References in superscript (a) Xu et al., (2012), (b) Moran et al., (2013), (c) Dragovic et al., (2006); (d) Easton et al., (2000).
Hsp110s have been found to prefer aromatic substrates in contrast to canonical Hsp70s which prefer aliphatic substrates. Sequence alignments of PfHsp70-z\_SBD show the binding residues flanked by the loops L\_1,2 and L\_3,4 (Figure 2.2). These residues are thought to be responsible for imparting substrate specificity (Xu et al., 2012). The PfHsp70-z substrate binding cleft is in the same orientation as with yeast and human Hsp110s (Xu et al., 2012). The sequence on the loops are in reverse orientation when compared to canonical Hsp70s. PfHsp70-z loop L\_1,2 HNININDASKS (Figure 2.2) versus MGG in Hsp70s and loop L\_3,4 KGH (Figure 2.2) versus TAEDNQS. These findings are in line with previous studies suggesting that the loops residues are conserved in Plasmodial Hsp110s (Xu et al., 2012). This suggests that PfHsp70-z possibly has similar aromatic substrate preference as other Hsp110s.

Apart from its interaction with substrate the PfHsp70-z\_SBD is also thought to interact with other proteins to form functional complexes. The residues that are implicated in interaction of Hsp110\_NBD with Hsp70\_NBD during nucleotide exchange events are less conserved in PfHsp70-z (Figure 2.2) (Moran et al., 2013; Dragovic et al., 2006). This suggests that Hsp110 may exhibit species specific nucleotide exchange function. PfHsp70-z is predicted to possess a protein-protein interaction Magic motif (Figure 2.2) and Tedwlyee motifs (Figure 2.2) (Easton et al., 2000). Magic and Tedwlyee motifs are the islands with high sequence conservation along the Hsp110\_SBD sequences and their function is enhanced by the residues implicated in protein-protein interactions (Figure 2.2). Furthermore, Hsp110s exhibit putative C-terminal protein-protein interaction motifs (Figure 2.2). Hsp110 possess D[L/M]D motif and the EEKDI motif on P. knowlesi, P. vivax and P. cynamolgi Hsp110 sequences which aligns to PEE residues on PfHsp70-z. The presence of several protein-protein interaction motifs suggests that PfHsp70-z interacts with Hsps as functional partners.

A high degree of homology in the cytosolic Hsp110 proteins was observed across plasmodial species with PfHsp70-z sharing the highest sequence identity with P. vivax homologue (68.4 %) (Appendix B1). However, PfHsp70-z exhibited a lower degree of conservation when compared to human and mouse orthologues (sequence identities of 22.4% and 22.2%,
respectively) and lowest to orthologues from *S. cerevisiae* 19.4% *T. brucei* 19.2%; *T. cruzi* 20.3%, and *Leishmania major* 19.7%, *L. braziliense* 18.9% (Appendix B1).

### 2.3.2 Phylogenetic analysis of PfHsp70-z and its homologues and orthologues

The phylogenetic analysis of PfHsp70z with its cytosolic homologues was conducted by comparison of the amino acid sequences. Plasmodium Hsp110 homologues are phylogenetically closely related as they cluster in a clade that is different from other species. Human and mouse Hsp110 cluster in a clade related to yeast. The TriTryps form two distinct clades that are *Trypanosomes* forming their own clade separate from the *Leishmania spp* (Figure 2.3).

![Phylogenetic analysis of Hsp110 homologues](image)

**Figure 2.3: Phylogenetic analysis of Hsp110 homologues**
The neighbour joining phylogenetic tree of Hsp110 homologues. Dendrograms were constructed using BioEdit (www.mbio.ncsu.edu/Bioedit/bioedit.html) Protdist neighbor phylogenetic analysis tool. The TreeView (Page 1996) option housed on the bioedit programme was used to generate dendrograms (Hall 1999). Boot strap was set at 100. The numbers represent distances. The accession numbers for the proteins are described in figure 2.1.

### 2.3.3 Generation of PfHsp70-z homology model

Homology modelling of PfHsp70-z subdomains was conducted using Sse1 crystal structures (3c7n.1.A.pdb; Polier *et al.*, 2008) as templates for the respective domains. To generate PfHsp70-zNBD three dimensional models, 50 templates were used and a model was built (Figure 2.4). The general Hsp110NBD subdomains IA, IB, IIA, IIB are conserved. Alignment of
the Hsp110\textsubscript{NBD} subdomains show structural similarities when aligned to Sse1 subdomains (Figure 2.4A; B).

<table>
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<tr>
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<tr>
<td>IB</td>
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**Figure 2.4:** A sequence alignment and homology model of the nucleotide binding domain of PfHsp70-z

Multiple sequence alignment between PfHsp70-z and Sse1 (A). PfHsp70-z amino acids 1-420 which constitute the putative nucleotide domain were aligned with Sse1 amino acids 1-396 which represent the NBD (Oh et al., 1999). (B) The Homology model of PfHsp70-z. Areas highlighted with ball and stick important for ATP binding in the ATP binding cleft between subdomains IA, IB, IIA and IIB. The two beta sheets highlighted in purple are the insertion segment on the IIB subdomain Sse1 as template (3c7n.1A.pdb; Polier et al., 2008). (C) The Homology model of Sse1\textsubscript{NBD} using template (3c7n.1A.pdb; Polier et al., 2008). (D) The Chimera matchmaker rendering PfHsp70-z onto Sse1 using Needleman-Wunsch alignment algorithm. The PfHsp70-z model is colored in blue and Sse1 model colored in brown.
The PfHsp70-z\textsubscript{NBD} three dimensional model is similar to the known Sse1p\textsubscript{NBD} which has a conserved nucleotide binding cleft similar to actin ATPase domain (Eason \textit{et al}., 2000; Polier \textit{et al}., 2008). There is structural conservation on PfHsp70-z nucleotide pore formed between subdomain IB and IIB (Figure 2.4B). This pore is thought to be responsible for the movement of nucleotide in and out of the binding cleft (Schuermann \textit{et al}., 2008). The presence of PfHsp70-z\textsubscript{NBD} insertion on IIB subdomain results in outstretched beta sheets (Figure 2.4B); which become more prominent when PfHsp70-z\textsubscript{NBD} is matched with Sse1\textsubscript{NBD} (Figure 2.4C; 2.4D; Polier \textit{et al}., 2008). These insertions may have evolved as a mechanism of PfHsp70-z specialisation for possible interaction with PfHsp70-1\textsubscript{NBD} as a NEF. This is in agreement with predictive studies conducted on \textit{P. falciparum} genome suggesting that PfHsp70-z is possibly the sole cytosolic NEF for PfHsp70-1 (Shonhai \textit{et al}., 2007; Bhartiya \textit{et al}., 2015). The structural predictions suggest that PfHsp70-z has possibly evolved to be a specialised cytosol localised NEF for PfHsp70-1.

The three dimensional model of PfHsp70-z\textsubscript{SBD} was generated using 80 templates (Figure 2.5). The general subdomain arrangement for Sse1\textsubscript{SBD} is conserved in PfHsp70-z\textsubscript{SBD} except the insertion of two beta sheets (\textbeta 6, \textbeta 10) (Figure 2.5A). The insertion may possibly suggest that PfHsp70-z exhibits better holdase function as it has more beta sheets which facilitate substrate interaction. Furthermore, the PfHsp70-z loop L\textsubscript{1,2} in the substrate binding domain exhibits an insertion which is bigger than that of the yeast and human homologues. This suggests that PfHsp70-z possibly interacts with larger substrates as compared to its yeast and human equivalents. This may imply that PfHsp70-z is more effective at suppressing protein aggregation compared to yeast and human equivalence (Muralidharan \textit{et al}., 2012). Based on observations made on PfHsp70-z\textsubscript{SBD} multiple sequence alignments (Figure 2.2), the predicted residues implicated in substrate binding are located in \textbeta 1; \textbeta 3 and \textbeta 4 (Figure 2.5B insert) (Polier \textit{et al}., 2010).
Figure 2.5: A sequence alignment and homology model of the substrate binding domain of PfHsp70-z

Multiple sequence alignment between PfHsp70-z and Sse1 (A). PfHsp70-z residues 421-728 constitute the putative substrate binding domain aligned with Sse1 residues 391-631 which represent the SBD. Areas highlighted are important for substrate binding residues (Red), substrate binding loops (Purple); Magic motif (Blue) Tedwyee motif (Green). The three dimensional model of PfHsp70-z (B). The highlighted areas are color coded similar to motifs and residues in (A). Insert represent the substrate binding cleft magnification and residues implicated. The model was generated using Sse1 as template (3c7n.1.A.pdb; Polier et al., 2008; Schuermann et al., 2008).
2.3.4 PfHsp70-z secondary structure prediction

The predictions of a three dimensional homology model for the last 143 residues of the C-terminus were unsuccessful. This may be as a result of this region being predominantly unstructured. Therefore, the PfHsp70-z secondary structure prediction was conducted using an online tool, PHYRE2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) (Figure 2.6). The secondary structure of PfHsp70-z was predicted to be composed of α-helices and disordered regions (Figure 2.6).

![Figure 2.6: Secondary structure prediction of PfHsp70-z](image)

Prediction of the secondary structure of full length PfHsp70-z, Sse1 and HSPH1 was conducted using both PHYRE2. Sequences were retrieved from PlasmoDB and NCBI and the accession numbers are as listed in Figure 2.1.

2.3.5 Composite analysis of the amino acid sequence of PfHsp70-z and design of peptide antibody

Knowledge of the peptide regions of immunogenicity is essential for peptide based antibody design. Composite analysis of PfHsp70-z was conducted using DNASTAR to determine a unique peptide sequence along the protein (Figure 2.7). Thirteen putative epitopes were predicted by GenScript Antigen Design Tool, OptimumAntigenTM design tool. Seven putative peptides were located in the ATPase domain and four within the PfHsp70-z580 and three were located on the C-terminal domain (Appendix B2). The peptide 768-782 (CSLQEQEKNKPLYEP)
was selected because it exhibited the lowest degree of sequence conservation in addition to it being surface exposed, possessing appropriate charge, hydophilicity and antigenic indices (Figure 2.7). After the successful design of the antigenic peptide, BLAST analysis was performed on the sequence and no significant similarities to *E. coli*, human, rabbit, goat and mouse protein sequences was detected. The selected peptide sequence was submitted to Genscript (USA) for the antibody production in a rabbit. The antibody was used for subsequent analysis (Section 3.3.4; 5.3.1; 5.3.2; 5.3.3; Zininga *et al*., 2015a).

![Composite analysis of full length PfHsp70-z amino acid sequence](image)

**Figure 2.7: Composite analysis of full length PfHsp70-z amino acid sequence**
Antigenicity of PfHsp70-z was analysed through the composite analysis of the amino acid sequence using DNASTAR-Proteon 3D. Positioning of residues are specified by numbers along the x-axis. The position of the selected peptide is indicated by a red box, along with the peptide sequence below it. Arrows below the graph indicate the relative positions of the domains of PfHsp70-z. (A, B, C) *Chou-Fasman* – predicts secondary structure (Chou and Fasman, 1978); (D, E) *Kyte-Doolittle hydropathy* – plots hydropilicity and hydrophobicity plots (Kyte and Doolittle, 1982); (F) *Lehninger* predicts the surface charge and plots Positive and negative charge plots (Lehninger, 2005). (G) *Karplus- Schultz* – predicts chain flexibility and plots enhanced flexibility as a peak (Karplus and Schultz, 1988). (H) *Jameson-Wolf antigenic index* – computes an overall antigenicity analysis by combining all the previous analyses, epitopic regions are plotted as peaks. The analysis was conducted using Proteon 3D on DNASTAR Version 12.2.0.80.

### 2.3.6 Genomic neighbourhood of PfHsp70-z

Eukaryotic genes are thought to be regulated in groups as gene clusters/genomic neighbourhoods based on shared distinct transcriptional activity (De and Babu, 1999). These events are known to affect the expression of several genes simultaneously and as such genes
within PfHsp70-z neighbourhood may be under cis-regulatory elements such as transcription factors. The PfHsp70-z gene is located on chromosome 7, on P. falciparum genome (Figure 2.8A). The members of the gene neighbours of PfHsp70-z show similarities with gene neighbours of Hsp110 from other plasmodial species (Figure 2.8B).

**Figure 2.8: Chromosomal location of PfHsp70-z gene**

The chromosomal location of the gene coding for Hsp70-z on Plasmodia species. PfHsp70-z location on P. falciparum chromosome 7 spanning from 380 kbp - 422 kpb (A). The chromosomal location of the plasmodium homologues of PfHsp70-z (B). Gene code blue arrows and those coded in complement are shown in red arrows, adapted from PlasmoDB.

PfHsp70-z gene neighbours include PfHsp90 (PF3D7_0708400) and the chloroquine resistance transporter (CRT; PF3D7_0709000) genes (Figure 2.8). This suggests that among others PfHsp90 and Pfcrt genes are possibly regulated simultaneously with PfHsp70-z.

**2.3.7 Predicted interaction partners of PfHsp70-z**

PfHsp70-z network data was retrieved from STRING database and analysed using Cytoscape (Shannon et al., 2004). Betweenness centrality and closeness centrality analyses were conducted using Cytoscape NetworkAnalyzer tool (Brandes, 2001). The node sizes and closeness suggest that PfHsp70z functionally interacts mostly with its interactors in the
following order: PF3D7_0818900 (PfHsp70-1) > PF3D7_071437900 (PfHsp40) > PF3D7_0708400 (PfHsp90) > PF3D7_1434300 (PfHop) > PF3D7_0517700 (Eukaryotic translation initiation factor) (Figure 2.8). Mapping of the interactome was conducted based on weighted evidence from direct interaction evidence from *P. falciparum*. The threshold for interactions was set at a confidence level of 0.7. This suggests that the predicted interactions exhibit a set at confidence level which is above 0.7 probability of occurrence (Jensen *et al.*, 2009).

![Graph showing predicted direct interaction partners](image)

**Figure 2.9: PfHsp70-z predicted direct interaction partners**

The predicted interactome of PfHsp70-z reflect interaction likelihood using betweenness centrality and closeness centrality. The node size was mapped to betweenness centrality of PfHsp70-z reflecting the amount of control it exerts over the interactions of other proteins in the network (Yoon *et al.*, 2006). The node colours were mapped to closeness centrality a measure of communication thus showing how fast (red) or slow (green) information spreads from PfHsp70-z to other reachable nodes in the network (Newman, 2003). The edges represents functional clusters as the red edges (line) represents Hsp40s, Brown edges represent translation factors, blue edges represent known molecular chaperones and co-chaperones forming a complex with Hsp90, Purple edges represent interaction with plasmoidal conserved proteins; green edges represent ubiquiting factors, and sky blue edge represent Hsp90 phosphorylation factor.
2.4 Discussion

The bioinformatics analyses conducted in this study unravelled PfHsp70-z as a structurally divergent Hsp110 protein. PfHsp70-z is predicted to be an NEF of PfHsp70-1 (Shonhai et al., 2007). In order to carry out this function, PfHsp70-z must be able to bind nucleotides. This study, using multiple sequence alignments, uncovered the general conservation for residues that are required for ATP binding in PfHsp70-z (Figure 2.1). Based on the three dimensional structure of PfHsp70-z (Figure 2.4), PfHsp70-z possibly functions as a crow bar to open Hsp70\textsubscript{NBD} IIB and IB subdomains thereby releasing ADP trapped in the Hsp70\textsubscript{NBD} binding cleft (Dragovic et al., 2006).

The orientation of the predicted three dimensional structure of the substrate binding cleft is conserved in PfHsp70-z (Figure 2.5). The loop L\textsubscript{1,2} in PfHsp70-z in the substrate binding is extended due to the insertion NDAS on loop L\textsubscript{1,2}. It is plausible that the insertion may have arisen in order to increase the size of the substrate interaction site by enabling PfHsp70-z to bind bigger substrates. This explains PfHsp70-z’s competitive advantage as chaperone over human and yeast homologues (Muralidharan et al., 2012). Taken together, PfHsp70-z is predicted to possibly function as a robust chaperone with preference for aromatic substrates as previously observed (Muralidharan et al., 2012).

PfHsp70-z is predicted to functionally interact with other proteins by the presence of conserved Magic motif and the Tedwlyee motif (Easton et al., 2000), that are thought to be involved in protein-protein interaction (Figure 2.2). These two motifs are thought to be at the centre of protein-protein interaction (Easton et al., 2000). Interestingly, the conserved Tedwlyee motif is also thought to facilitate the interaction with Hsp70 during NEF function. These data suggest that PfHsp70-z interacts with PfHsp70-1 through both its NBD and SBD in conducting its role as a NEF of PfHsp70-1.
Chapter 2: Bioinformatics Analyses of the Structural Features of PfHsp70-z

Hsp110 proteins exhibit unique linker segments that connect the N-terminal Hsp110NBD to the C-terminal Hsp110SBD (Polier 2008). It has been suggested that the linker of Hsp110 is fairly rigid, resulting in the protein exhibiting reduced allosteric function (Liu and Hendrickson, 2007). Based on sequence alignment, Plasmodial Hsp110 proteins exhibit a linker segment that differs from that of canonical Hsp70s (Figure 2.1). Furthermore, the linker of PfHsp70-z is divergent from that of human and yeast Hsp110s (Figure 2.1). Since the linker region is required for Hsp70 allosteric function (Vogel et al. 2006), the linker of plasmodial Hsp110 may confer specific functional features to these proteins. Indeed, it has been proposed that Hsp110 from yeast may possess unique structural features compared to the human homologue (Raviol et al., 2005). It remains to be experimentally validated, the effects of nucleotide binding assays on the structural conformation of PfHsp70-z (Sections 4.3.1; 4.3.3).

PfHsp70-z has been predicted to associate mostly with chaperones in the following order: PfHsp70-1 > PfHsp40 (PF3D7_1437900) > PfHsp90 (PF3D7_0708400) > PfHop (PF3D7_1434300) (Figure 2.9). Phosphorylation regulates the chaperone function of some chaperones. For example, the inhibition of PfHsp90 phosphorylation results in non-functional PfHsp90 (Banumathy et al., 2003). The TPR rich phosphatase PP5 (PF3D7_1355500), a known Hsp90 phosphatase (Dobson et al. 2001; Kumar et al. 2003) was predicted to interact with PfHsp70-z. This interaction is possibly through the formation of chaperone complexes where PfHsp70-z occurs in complex with PfHsp70-1, PfHsp90-PfHop and it remains to be experimentally validated. Chloroquine resistance in P. falciparum is mapped to a 36 kb segment of Chromosome 7, which includes the PfHsp90 and PfHsp70-z (PfCg4) gene (Figure 2.8; Su et al., 1997). The gene location data suggest that PfHsp70-z may be under the same regulatory mechanism with Chloroquine resistance transporter (CRT) genes. Furthermore, it has previously been demonstrated that the expression of yeast Hsp90 and Hsp110 are both induced by heat shock transcription factor carboxyl terminal activation domain (HSF-CTD) (Lui et al., 1999). The induction of PfHsp70-z and PfHsp90 is possibly important for cell cycle progression during heat stress conditions. Altogether, this suggests PfHsp70-z and PfHsp90 potential cooperation may facilitate parasite survival during heat stress conditions.
The interactome data suggest that PfHsp70-z is a potential NEF for PfHsp70-1 as it is the only cytosolic NEF homology (Bhartiya et al., 2015). It has been established that PfHsp70-1 interacts with PfHsp90 through PfHop as the adaptor protein (Banumathy et al., 2003; Gitau et al., 2012; Zininga et al., 2015b). Based on the interactome data, this study predicts the possible interaction of PfHsp70-z with the PfHsp70-1-PfHop-PfHsp90 complex (Figure 2.9; Gitau et al., 2012). However, the role of PfHsp70-z in this complex remains to be validated.

Data from this study predicts PfHsp70-z to interact with Hsp40s (Figure 2.8). It is known that PfHsp40 (PF3D7_1437900) facilitates PfHsp70-1 functional cycle (Figure 1.8; Botha et al., 2011). The predicted functional cooperation of PfHsp70-z and PfHsp40 possibly regulate PfHsp70-1 functional cycle.

PfHsp70-z is also predicted to interact with peptidyl-prolyl cis-trans isomerase (PPI) (PF3D7_0803000) which catalyzes the cis-trans isomerization of proline imidic peptide bonds in oligopeptides (Hall et al., 2002). PfHsp70-z is possibly involved in the formation of disulphide bond during protein folding through its interaction with PPIs. Furthermore, PfHsp70-z is predicted to interact with proteins involved in translational initiation (Tuteja and Pradhan, 2009). This suggests that PfHsp70-z is involved in virtually every step of a protein life by interacting with translation factors; chaperones and ubiquitin dependant protein degradation factors (Figure 2.9). However, it remains to be validated if the predicted interactors are functional partners or potential substrates.

The aim of this study was to utilize available bioinformatics tools to glean the role of PfHsp70-z. PfHsp70-z functional features were mapped and PfHsp70-z interact-actors were established. PfHsp70-z is fairly divergent from human homologue. The divergence makes it prospect for antimalarial possible drug target discovery.
CHAPTER 3

PRODUCTION OF RECOMBINANT PFHSP70-Z PROTEIN AND
ANALYSIS OF ITS SECONDARY AND TERTIARY STRUCTURES
Chapter 3

Production of Recombinant PfHsp70-z Protein and Analysis of its Secondary and Tertiary Structures

3.1 Introduction

Hsp110s belong to an Hsp70 subfamily of eukaryotic chaperones that have been implicated in a variety of cellular functions. Hsp110 like Hsp70, can recognise and bind hydrophobic peptide sequences and exhibits ATPase activity (Goeckeler et al., 2008). However, Hsp110s cannot refold denatured substrates and act as ‘holdases’ binding denatured substrates and suppress substrate aggregation (Oh et al., 1997; Goeckeler et al., 2002). Specific intracellular functions of Hsp110 are poorly characterised (Moran et al., 2013). Hsp110s are thought to serve as NEFs of their respective canonical Hsp70 counterparts (Dragovic et al., 2006; Andreasson et al., 2008). Nucleotide exchange indirectly determines the substrate dwell time on Hsp70SBD thereby influencing substrate fate (Mandal et al., 2010). The premature release of substrates from Hsp70 could result in their aggregation, and ultimate degradation (Mayer and Bukau, 2005). NEFs from prokaryotes and eukaryotes are structurally unrelated but they serve a common role (Raviol et al., 2005). In *P. falciparum* only PfHsp70-z, an Hsp110 homologue, is based in the cytosol and thus may function as a potential NEF of the well characterized canonical Hsp70 chaperone, PfHsp70-1 that also occurs in the cytosol (Shonhai et al., 2007). Although PfHsp70-z is thought to play an essential role through suppressing aggregation of malarial asparagine repeat-rich proteins (Muralidharan et al., 2012), it is possible that its function as NEF of PfHsp70-1 may be crucial for parasite survival. Therefore, this study sought to heterologously express and purify recombinant PfHsp70-z and to investigate its biophysical characteristics.

The objectives of this study were to:

i) Heterologously express and purify recombinant PfHsp70-z protein;

ii) Determine the secondary and tertiary structure of recombinant PfHsp70-z protein;

iii) Assess the heat stability of recombinant PfHsp70-z;

iv) Explore the formation of higher order oligomers by recombinant PfHsp70-z; and

v) Determine the ATPase activity of recombinant PfHsp70-z protein.
3.2 Experimental Procedures

3.2.1 Materials

The following plasmids used are summarised in Table 3.1: pQE30 vector (Qiagen, Germany) and pQE30/PfHsp70-z plasmid expressing PfHsp70-z (Zininga et al., 2015a); pQE30/PfHsp70-1 plasmid expressing PfHsp70-1 (Matambo et al., 2004; Shonhai et al., 2005); pQE30/PfHsp70-1_{NBD} plasmid expressing the PfHsp70-1_{NBD} (Zininga et al., 2015b). The following antibodies were used: the α-PfHsp70-z antibody produced in rabbit against peptide: CSLQEQEKNKPLYEP, corresponding to amino acids 768–782 of the PfHsp70-z amino acid sequence was secured from GenScript (USA) (section 2.3.5; Zininga et al., 2015a). Polyclonal rabbit raised antibodies specific for PfHsp70-1 that were previously described (Pesce et al., 2008) were used to validate the authenticity of recombinant PfHsp70-1 protein. The rest of the reagents used in the study are listed in Appendix C.

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3.2.2 Construction of plasmid expressing PfHsp70-z

Codon harmonized form of the gene for *PfHsp70-z* (PlasmoDB accession number: P3D7_088000) was made by Genscript. The DNA segment encoding regions of PfHsp70-z were
subsequently PCR amplified from pUC57/PfHsp70-z using the forward primer (5’–CATCACGGATCCATGAGC–3’) with a BamHI restriction site and reverse primer (5’–GCTAATTAAGCTTCCATTCTCCGG – 3’) with a HindIII restriction site. The gene was subsequently cloned in pQE30 (Qiagen) making pQE30/PfHsp70-z construct used to express PfHsp70-z (Zininga et al., 2015a) and was verified by DNA sequencing. The pQE30/PfHsp70-z plasmid constructs was purified using the Zymo Research Plasmid Miniprep™ kit (Epigenetics, U.S.A) according to manufactures instructions (Appendix A1). The DNA was digested (Appendix A2) using restriction enzymes, HindIII and BamHI (Thermo Scientific, U.S.A). The restriction products were then analysed using 0.8 % agarose gel electrophoresis (Appendix A3).

3.2.3 Confirmation of PfHsp70-1 and PfHsp70-1\textsubscript{NBD} DNA constructs

A plasmid construct expressing PfHsp70-1 (PlasmoDB accession number: PF3D7_0818900) (pQE30/PfHsp70-1) was used to express PfHsp70-1 as previously described (Matambo et al., 2004; Shonhai et al. 2008). Another plasmid construct (pQE30/PfHsp70-1\textsubscript{NBD}) expressing the PfHsp70-1\textsubscript{NBD} was used to express PfHsp70-1\textsubscript{NBD} (Zininga et al., 2015b). To confirm the integrity of the pQE30/PfHsp70-1 and pQE30/PfHsp70-1\textsubscript{NBD}, the plasmid constructs were purified as previously described (section 3.2.2) and was digested (Appendix A2) using restriction enzymes, HindIII + BamHI for pQE30/PfHsp70-1 and BamHI + SpeI for pQE30/PfHsp70-1\textsubscript{NBD}, respectively (Thermo Scientific, U.S.A). The restriction products were then analysed using 0.8 % agarose gel electrophoresis.

3.2.4. Expression of recombinant proteins

Competent \textit{E. coli} JM109 (DE3) cells were chemically transformed with pQE30/PfHsp70-z (Appendix A4; A5; Zininga et al., 2015a). Competent \textit{E. coli} XL1 Blue cells where chemically transformed with either pQE30/PfHsp70-1 or pQE30/PfHsp70-1\textsubscript{NDB} (Appendix A4; A5; Shonhai et al., 2008; Zininga et al., 2014b). The colonies carrying the plasmids were respectively grown for 12 hours in 50 ml 2X yeast/tryptone (2YT) (tryptone 16 g/L, yeast extract 10 g/L and NaCl 5 g/L) broth containing 100 \(\mu\)g/ml ampicillin at 30 °C, then
subsequently diluted using 450 mL 2YT. The cells were allowed to grow at 30 °C with shaking at 160 rpm using FMH 200 shaker (FMH Electronics, RSA). At A_{600} = 0.6, the expression was induced using 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG). After growing for more than 24 hours, the cells were harvested by centrifugation at 5000 g for 20 minutes at 4 °C and the pellet was suspended in lysis buffer (100 mM Tris–HC1, pH 7.4, 300 mM NaCl, 10 mM Imidazole containing 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mg/ml lysozyme) at 23°C for 1 hour. The cell lysates were kept at -80 °C for storage. Protein expression samples were analyzed using 12 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualised by Coomassie blue (Appendix A6). The production of the His_{6}-tagged recombinant protein was confirmed by Western analysis using mouse monoclonal anti-His_{6}-horseradish peroxidase antibodies (α-His) [1 : 2000 dilution] (Sigma-Aldrich, U.S.A). The production of recombinant proteins was confirmed by Western blot (Appendix A.7) using rabbit-raised polyclonal anti-PfHsp70-z (α-PfHsp70-z) [1 : 2000 dilution] as primary antibody for PfHsp70-z. PfHsp70-1 was confirmed using anti-PfHsp70-1 (α-PfHsp70-1) [1: 2000 dilution] as primary antibody for full length and truncated PfHsp70-1_{NBD}. The secondary antibody used was goat raised anti-rabbit horseradish peroxidase conjugated antibody (1: 5000 dilution). Imaging of the protein bands on the Western blot was conducted using the ECL kit (Thermoscientific, USA) as per manufacturer’s instructions (Appendix A8). Images were captured using ChemiDoc Imaging system (Bio-Rad, USA).

3.2.5 Purification of recombinant proteins

The harvested JM109 and XL1 Blue E. coli cells stored at – 80 °C were retrieved and allowed to thaw on ice for one hour. Nucleic acids were precipitated by addition of 0.1 % (w/v) Poly (ethyleneimine) (PEI). The cell lysate was then centrifuged at 5000 g for 20 minutes at 4°C. The supernatant was loaded onto a HisPur™ Nickel-charged nitritotriacetic acid (Ni-NTA) (ThermoScientific) immobilized metal affinity chromatography column (IMAC) to allow His_{6}-PfHsp70-z/PfHsp70-1/PfHsp70-1_{NBD} in the soluble cell extract to bind to HisPur™ Ni-NTA at 4°C for four hours. The HisPur™ Ni-NTA IMAC column was then washed using wash buffer
Production of Recombinant PfHsp70-z Protein and Analysis of its Secondary and Tertiary Structures

(100 mM Tris-HCl, pH 7.4, 300 mM NaCl, 25 mM Imidazole, 1 mM EDTA containing 1 mM PMSF). The bound protein was eluted using elution buffer (100 mM Tris-HCl, pH 7.4, 300 mM NaCl, 500 mM Imidazole, 1 mM EDTA, and containing 1 mM PMSF). The protein was extensively dialysed using SnakeSkin tubing 10,000 MWCO (ThermoScientific) in dialysis buffer (100 mM Tris-HCl, pH 7.4, 300 mM NaCl, 10 mM Imidazole, 10 % (v/v) glycerol, containing 1 mM PMSF) and storage buffer buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 % (v/v) glycerol, containing 1 mM PMSF). The purity of the eluted protein was assessed using SDS-PAGE and further confirmed by Western blotting as previously described (section 3.2.5). To validate that the purified PfHsp70-z protein was not contaminated with traces of *E. coli* Hsp70 (DnaK), the protein preparations were probed by Western blotting using α-DnaK antibodies (Stressgen, Germany). The protein was subsequently concentrated with polyethylene glycol. Protein concentrations were estimated by Bradford assay (Sigma-Aldrich) following manufacturer’s instructions using bovine serum albumin as protein standard (Appendix A9). The protein concentrations were further confirmed by using the christoph-leidig equation (Appendix A10).

3.2.6 Investigation of the secondary structural organisation of PfHsp70-z

The secondary structure of recombinant PfHsp70-z was analysed using Far-UV circular dichroism (CD). CD spectroscopy experiments were performed using a J-1500 CD spectrometer (JASCO Ltd, UK) equipped with a Peltier temperature controller. PfHsp70-z recombinant protein (0.2 μM) was suspended in 20mM Tris-HCl pH 7.4, 100mM sodium fluoride buffer and analysed using a 0.1 cm path-length quartz cuvette (Hellma). Spectra were averaged for least 15 scans after baseline correction (subtraction of spectrum from buffer in which PfHsp70-z protein was excluded). The CD measurements were normalised to protein concentration and presented as molar ellipticity, (θ) deg.cm².dmol⁻¹ (Appendix A11). Secondary structure predictions were conducted on the observed CD spectrum at constant temperature set at 19 °C. The spectra were deconvulted to α-helix, β-sheet, β- turn and

The effect of heat and urea on PfHsp70-z was investigated, as thermal and chemical denaturants, respectively. The secondary structure of the protein was monitored at 222 nm as the temperature was raised monotonically initially from 19 °C to 65 °C at a rate of 0.5 °C per min. The temperature was restored to 19°C. The experiment was repeated, this time the temperature was raised from 19 °C to 95 °C at a rate of 0.5 °C. The folded state of the PfHsp70-z protein at any given temperature was determined as follows (Misra and Ramachandran, 2009):

\[
\frac{((\theta)_t - (\theta)_h)}{((\theta)_t) - (\theta)_l) \] \hspace{1cm} \text{Equation 1}
\]

Where \((\theta)_t\) is the molar ellipticity at any given temperature, \((\theta)_h\) at highest temperature, and \((\theta)_l\) at lowest temperature, respectively. The effects of urea on the stability of PfHsp70-z was monitored by assessing the molar residue ellipticity at 222 nm, with temperature set at 19 °C in the absence and presence of varying urea concentration (1 M to 10 M). The folded state of PfHsp70-z protein exposed to various levels of urea was calculated using the Equation 1, described above by substituting the respective urea concentrations for the temperature values.

3.2.7 Investigation of the tertiary structural organisation of PfHsp70-z

Further to the assessment of secondary structure using CD, the tertiary structural organisation of PfHsp70-z proteins was assessed using tryptophan fluorescence. This was conducted by monitoring the changes in tertiary structure of the protein that was subjected to urea denaturation by conducting tryptophan fluorescence based analysis. The recombinant PfHsp70-z protein (0.45 μg/ml) was incubated in assay buffer A (25 mM HEPES-KOH pH 7.5, 100 mM KCl, 10 mM MgOAc) for 20 minutes at 20°C in the presence of the variable urea concentrations (1 M- 8 M). Fluorescence spectra were recorded between 320 nm and 450 nm.
after initial excitation at 295 nm using JASCO FP-6300 spectrofluorometer (JASCO, Tokyo, JAPAN).

3.2.8 Assessment of the capability of PfHsp70-z to form higher order oligomers

The capability of PfHsp70-z molecules to self-associate was conducted using a Proteon XPR36 (BioRad, USA) surface plasmon resonance (SPR) machine. The assays were conducted at room temperature (25 ℃), filter sterilised and degassed PBS-Tween (10 mM Phosphate, 137 mM NaCl, 3 mM KCl, 0.005 % (v/v) Tween 20 and 20 mM EDTA; pH 7.4) was used as running buffer. The immobilisation of ligand was achieved through covalent attachment to the modified alginate polymer layer on the GLC sensor chip via amine coupling following a protocol that was provided by the manufacturer (BioRad, USA) (Appendix A12; B4; B5; B6). PfHsp70-z (as ligand) was immobilised at concentrations of 0.5 µg/mL and 1 µg/mL. At these concentrations 187 response units (RU) was achieved for PfHsp70-z per immobilization surface (Moriaty, 2010). As analytes aliquots of PfHsp70-z and BSA (as negative control) were prepared at final concentration of 2000 nM, 1000 nM, 500 nM, 250 nM, and 125 nM and were injected at 50 μl/min into each horizontal channel at a rate of 50μl/min. The analysis was conducted in the absence or presence of 5 mM of either ATP or ADP. Association was allowed for 2 minutes, dissociation was monitored for 8 minutes. The SPR sensorgrams for dimerization were fit to a simple Langmuir kinetic binding model to calculate the association rate constant ($k_a$ in the unit of M$^{-1}$s$^{-1}$), dissociation rate constant ($k_d$ in the unit of s$^{-1}$) and the equilibrium constant ($K_D$ in the unit of M). Data analysis was contacted after subtraction of the baseline RU (Buffer with ATP/ADP with BSA and without analyte protein). Association rate constant, dissociation rate constant and equilibrium constant data was processed and analysed using ProteOn Manager™ software version 3.1.0.6 by concatenating the responses of all five analyte concentrations (O’Shannessy et al., 1993).
3.2.9 Investigation of ATPase activity of PfHsp70-z

The ATPase activity of PfHsp70-z was determined by quantifying the amount of released inorganic phosphate based on a previously described approach (Matambo et al., 2004) with modifications. Briefly, 0.4 μMPfHsp70-z was incubated for five minutes in buffer HKMD (10 mM HEPES-KOH pH 7.5, 100 mM KCl, 2 mM MgCl₂, 0.5 mM DTT). The reaction was started by addition of ATP and samples were collected after every 30 minutes for four hours and reaction was stopped with 10% (w/v) SDS and using 1.25 (w/v) % ammonium molybdate in 6.5 % H₂SO₄ and 9 % (w/v) ascorbic acid to quantitate the release of inorganic phosphate. The reaction mix without the protein was used as a non-enzymatic control. Samples were read at 660nm using SpectraMax M3 spectrometer (Molecular Devices, U.S.A). The absorbance values were extrapolated against a standard calibration curve using Na₂HPO₄ as standard (Appendix B7). The basal ATPase activity was expressed as nmol Pi released/min/mg of recombinant protein. Kinetic plots for the ATPase activities of PfHsp70-z and PfHsp70-1 were determined by generating Michaelis–Menten plots using GraphPad Prism 6.05. At least three independent batches of recombinant protein were used in separate experiments.
3.3 Results

3.3.1 Confirmation of the pQE30/PfHsp70-z plasmid

The integrity of pQE30/PfHsp70-z plasmid construct was verified by sequencing and restriction digest (Figure 3.1). Restriction with either BamHI or HindIII resulted in linearised plasmid that migrated at 6046 bp (Figure 3.1B). Double digestion using BamHI and HindIII resulted in fragments of 3419bp and 2627bp, corresponding to the pQE30 expression vector and PfHsp70-z insert sizes, respectively (Figure 3.1B).

3.3.2 Confirmation of pQE30/PfHsp70-1 plasmid

The integrity of pQE30/PfHsp70-1 was verified by restriction digest using BamHI and HindIII (Figure 3.2; Matambo et al., 2004). Single restriction site digestion with either BamHI or HindIII resulted in the migration of linearised plasmid of 5471bp (Figure 3.2B). Double digestion using BamHI and HindIII excised the PfHsp70-1 coding sequence (2627bp) resulting in fragments of 3419bp and 2052bp, corresponding to the pQE30 expression vector and PfHsp70-1 insert sizes, respectively (Figure 3.2B).
3.3.3 Confirmation of pQE30/PfHsp70-1\textsubscript{NBD} plasmid

The plasmid with the truncated version of PfHsp70-1 expressing the nucleotide binding domain pQE30/PfHsp70-1\textsubscript{NBD} (Zininga \textit{et al.}, 2015b) was verified by restriction analysis. Restriction enzymes \textit{Bam}HI and \textit{Spe}I were used for restriction analysis to confirm the integrity of the pQE30/PfHsp70-1\textsubscript{NBD} (Figure 3.3 B).

Figure 3.3: pQE30/PfHsp70-1\textsubscript{NBD} plasmid map and restriction agarose gel

Restriction analysis of pQE30/PfHsp70-1\textsubscript{NBD} DNA plasmid (A) Plasmid map of pQE30/PfHsp70-1\textsubscript{NBD} showing the \textit{Bam}HI and \textit{Spe}I restriction sites. (B) Agarose gel electrophoresis of pQE30/PfHsp70-1\textsubscript{NBD}: lane M, DNA molecular weight maker; lane 1, undigested pQE30/PfHsp70-1\textsubscript{NBD} plasmid; lane 2, pQE30/PfHsp70-1\textsubscript{NBD} digested with \textit{Bam}HI; lane 3, pQE30/PfHsp70-1\textsubscript{NBD} digested with \textit{Spe}I; lane 4, pQE30/PfHsp70-1\textsubscript{NBD} digested with \textit{Bam}HI and \textit{Spe}I.
Digestion with either BamHI or SpeI resulted in the linearised plasmid of 5471bp (Figure 3.3 B). The PfHsp70-1NBD construct (1201bp), was confirmed by digesting the plasmid with both BamHI and SpeI, which resulted in the DNA fragments of 4270bp and 1201bp (Figure 3.3 B).

3.3.4 Overexpression and purification of recombinant PfHsp70-z protein

Recombinant PfHsp70-z protein was successfully expressed in JM109 *E. coli* cells (Figure 3.4A). The PfHsp70-z production was analysed by SDS-PAGE and and confirmed by Western blot analysis using α-PfHsp70-z. SDS analysis of the samples retrieved during expression showed an increase in the level of expression of a species at approximately 100 kDa size. The control confirmed the absence of 100 kDa species in untransformed JM109 *E. coli* cells (Figure 3.4A). This suggests that the 100 kDa species were recombinant PfHsp70-z protein and this was confirmed by Western blot. The same 100 kDa species was detected from cells prior to induction (Figure 3.4A) indicating leak expression of the protein. The expression was successfully induced with IPTG (Figure 3.4A). Western analysis confirmed the leaky expression and shown some bands below 70kDa which are possibly breakdown products as their intensity increased in the overnight sample (Figure 3.4A).

PfHsp70-z expressed was purified by affinity chromatography (Figure 3.4B). Before purification it was important to investigate protein solubility the pellet and soluble fractions were analysed. The 100 kDa band in the soluble fraction was thicker than in the pellet fraction (Figure 3.4B). This suggests that recombinant PfHsp70-z protein was soluble and it was loaded on to the IMAC. Some of the PfHsp70-z protein was lost during washes (Figure 3.4B lane W). PfHsp70-z was eluted as a species of approximately 100 kDa (Figure 3.4B). Purifications of PfHsp70-z resulted in a final protein yield of approximately 9.0 µg/L of protein. However, a band running approximately above 200 kDa was also observed which was recognised by both α-His and α-PfHsp70-z antibodies. It was surmised that this band possibly represented a dimer form of PfHsp70-z (section 3.3.10). The purity of the eluted protein was further validated by probing using α-DnaK antibodies and confirmed the assessment that the eluted PfHsp70-z protein was free from DnaK contamination (Figure 3.4B).
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Figure 3.4: Expression and purification of recombinant PfHsp70-z

PfHsp70-z was expressed in E. coli JM109 cells transformed with pQE30/PfHsp70-z. SDS-PAGE (12%) and Western analyses of the expression (A) and purification (B) of recombinant PfHsp70-z; lane M—Page ruler (Thermo Scientific) in kDa; lane C—Total extract for cells transformed with a neat pQE30 plasmid; lane 0—total cell extract transformed with pQE30/PfHsp70-z prior to IPTG induction; lanes 2, 4, 6, O/N—total cell lysate obtained 2, 4, 6 and 16 h post induction, respectively. Lane P, S—pellet and soluble fractions obtained from the total lysate for cells transformed with pQE30/PfHsp70-z, respectively; lane F—flow through; lane W—wash samples, and lane E—PfHsp70-z protein eluted using 500 mM imidazole. Lower panels: Western blot based on use of α-PfHsp70-z and α-His antibodies confirming expression and purification of PfHsp70-z protein. To confirm the purity of PfHsp70-z from DnaK contamination, Western blotting was conducted using α-DnaK antibodies.

3.3.5 Overexpression and purification of recombinant PfHsp70-1 protein

The recombinant form of PfHsp70-1 protein was expressed and purified using previously described protocols (Shonhai et al., 2008). Full length PfHsp70-1 represented by a species of 74 kDa on SDS-PAGE analysis was over-expressed (Figure 3.5A) and purified from E. coli XL1-Blue cells (Figure 3.5B). Western blotting analysis using antibodies raised against PfHsp70-1 as well as antibodies that recognize the polyhistidine tag, detected PfHsp70-1 (Figure 3.5). The cells started expressing PfHsp70-1 before induction (Figure 3.5A) suggesting leaky
expression. The cells were able to produce more protein post-induction (Figure 3.5A), with optimum production at 16 hours (Figure 3.5A). Breakdown products of PfHsp70-1 were detected represented by bands of smaller species of 35 kDa, 50 kDa and 60 kDa observed in samples representing protein expression (Figure 3.5A). PfHsp70-1 was purified natively using affinity chromatography (Figure 3.5B).

Figure 3.5: Expression and purification of recombinant PfHsp70-1 protein
PfHsp70-1 was expressed in *E. coli* XL1 Blue cells transformed with pQE30/PfHsp70-1. SDS-PAGE (12 %) and Western analyses of (A) the expression of recombinant PfHsp70-1, (B) the purification of PfHsp70-1. Lane M – Page ruler (Thermo Scientific) in kDa is shown on the left hand side; lane C – the total extract for cells transformed with a neat pQE30 plasmid; lane 0 – total cell extract transformed with pQE30/PfHsp70-1; pQE30/PfHsp70-1_NBD prior to IPTG induction; lanes 2, 4, 6, O/N – total cell lysate obtained 2, 4, 6 hours and overnight post induction. Lane P, S-pellet and soluble fractions obtained from the total lysate for cells transformed with pQE30/PfHsp70-1, respectively; lane F: flow through; lane W —wash samples, and lane E— PfHsp70-z protein eluted using 500 mM imidazole. Lane E – protein eluted using 500 mM imidazole. Lower panels: Western blot confirming expression, purification of PfHsp70-1 based on using α-PfHsp70-1 and α-His antibodies.

Comparison of PfHsp70-1 pellet and soluble fraction (Figure 3.5B) suggests that PfHsp70-1 existed more in the soluble fraction than in the pellet fraction. A significant amount of
recombinant PfHsp70-1 protein was lost in the flow through (Figure 3.5B) and during the wash steps (Figure 3.5B). Upon purification of PfHsp70-1_NBD a yield of approximately 7.0 µg/L protein was obtained.

### 3.3.6 Overexpression and purification of recombinant PfHsp70-1_NBD protein

The truncated PfHsp70-1 (PfHsp70-1_NBD) was over expressed and purified from *E. coli* XL1 Blue cells. Western blotting analysis using antibodies raised against PfHsp70-1 as well as antibodies that recognize the polyhistidine tag detected the PfHsp70-1_NBD.

**Figure 3.6: Expression and purification of recombinant PfHsp70-1_NBD protein**
PfHsp70-1_NBD was expressed in *E. coli* XL1 Blue cells transformed with pQE30/PfHsp70-1_NBD. SDS-PAGE (12%) and Western analyses of (A) the expression of recombinant PfHsp70-1_NBD, (B) the purification of PfHsp70-1_NBD. Lane M – Page ruler (Thermo Scientific) in kDa is shown on the left hand side; lane C - the total extract for cells transformed with a neat pQE30 plasmid; lane 0 – total cell extract transformed with pQE30/PfHsp70-1_NBD prior to IPTG induction; lanes 4, O/N – total cell lysate obtained 4 hours and overnight post induction; and lane WI/I/II- wash samples and lane E - protein eluted using 500 mM imidazole. Lower panels: Western blot based on use of α-PfHsp70-1 and α-His confirming expression, purification of PfHsp70-1_NBD.
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The production of PfHsp70-1\textsubscript{NBD} as a species of 45 kDa was confirmed by SDS-PAGE analysis (Figure 3.6A) and Western blotting (Figure 3.6B). Before IPTG induction, a 45 kDa protein band appearing to represent PfHsp70-1\textsubscript{NBD} was observed (Figure 3.6A). This suggests leaky expression. However, upon adding IPTG, the cells were able to express PfHsp70-1\textsubscript{NBD} after 16 hour post induction, after which they were harvested (Figure 3.6A). PfHsp70-1\textsubscript{NBD} was purified natively using affinity chromatography (Figure 3.6B). Purification of PfHsp70-1\textsubscript{NBD} resulted in a final yield of approximately 8.0 µg/L of protein.

3.3.7 Secondary structural analysis of PfHsp70-z

To confirm the secondary structure of PfHsp70-z and to investigate the effect of heat stress on its stability, CD spectrophotometric analysis was conducted. The recorded signals (experimental) between 190 nm and 260 nm were superimposed to predicted signals obtained using Dichroweb (Figure 3.7A). The correlation between the two signals that were resolved using the CDSSTR method gave NRMSD values of approximately 0.002, representing a good correlation. The far UV spectra exhibited a positive peak centred at 194 nM and two negative depressions (trough) at 209 and 221 nM, respectively (Figure 3.7A). This spectrum represents the helical character of the recombinant protein. The deconvolution of the CD spectra revealed that full length PfHsp70-z is predominantly composed of α-helices (62 %), followed by β-strands (16 %), β-turns (8 %) and unordered (14 %). The CD spectra measurements were repeated whilst heating the protein to 95 °C (Figure 3.7B), in order to determine if the protein structure is perturbed by temperature changes. Secondary structure stability was subsequently monitored at 221 nm, as the protein was partially heated to 65 °C (Figure 3.7C). The temperature was subsequently restored to 19 °C, allowing the protein to revert to its initial spectral molar residue ellipticity level (Figure 3.7C). On the other hand, when the temperature was raised to 95 °C, the protein failed to regain its original molar residue ellipticity secondary structure (Figure 3.7C). Attempts to refold the protein by reducing the temperature to 19 °C subsequently after heating the protein to 95 °C the protein, failed to refold to its initial molar residue ellipticity level (Figure 3.7C).
The folded fraction assessment of recombinant PfHsp70-z protein showed that more than 60% of the protein still maintaining its folded state at 80 °C (Figure 3.7D). In a separate experiment, the secondary structure of PfHsp70z was monitored at 221 nm as urea concentration was increased to 10 M (Figure 3.7E). The protein was fairly stable in the presence of up to 6 M urea (Figure 3.7E). Upon increasing the concentration of urea beyond this level, PfHsp70z irreversibly lost its secondary structure (Figure 3.7E). Assessments of the
folded fraction showed that 60% of the protein maintained a folded state in the presence of up to 6 M urea (Figure 3.7F). This further demonstrated that the structural conformation of PfHsp70-z was perturbed by urea in a concentration dependent fashion (Figure 3.7E; F).

3.3.8: Tertiary structural organisation of PfHsp70-z

The tertiary structure of PfHsp70-z was evaluated using tryptophan based fluorescence analysis of the protein in the presence of variable levels of urea and variable temperature (Figure 3.8). The tryptophan fluorescence was monitored over different experimental setups. The spectrum reduced in intensity as temperature was raised. This suggests that the tryptophan fluorescence was quenched as the temperature was raised observed as a decrease in relative fluorescence intensity (Figure 3.8A). Analysis of the changes in tryptophan fluorescence peak showed a change in the peak wavelength in response to temperature increase. This change was observed to be following a red shift of fluorescence peak in response to temperature increase (Figure 3.8B). PfHsp70-z tertiary structure assessment in response to increasing urea concentration showed that the tryptophan fluorescence signal decreased with an increase in urea concentration. This suggests quenching of the relative fluorescence dependent on urea concentration (Figure 3.8C). The tryptophan fluorescence changes registered a red shift of emission with maximum peak at 350 nm corresponding to urea concentration of 6.5 M (Figure 3.8D). This finding mirrors the observation that was made by assessing the perturbation of the secondary structure of the protein in response to urea treatment (Figure 3.7E; F).
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Figure 3.8: Analysis of the tertiary structure of PfHsp70-z by tryptophan fluorescence
The fluorescence emission spectra was monitored at 320 - 450 nm after an initial excitation at 295 nm. The protein tryptophan fluorescence emission spectra was recorded for PfHsp70-z protein exposed to various temperatures (A) to assess the effect of temperature on the emission spectra. Assessment of the red shift of PfHsp70z exposed to different temperature on the emission spectra (B). The recombinant Pfhs70-z protein tryptophan fluorescence emission spectra was also recorded under various urea concentrations (C). Assessment of the red shift of PfHsp70z exposed to different urea concentrations on the emission spectra (D) (Zinninga et al., 2015a).

3.3.9 Assessment of the ATPase activity of PfHsp70-z
The basal ATPase activities of both PfHsp70-z and PfHsp70-1 were determined using a colorimetric assay measuring the rate of inorganic phosphate released during ATP hydrolysis (Matambo et al., 2004). The assay was conducted under variable initial ATP concentrations (up to 1500 μM). The Michaelis-Menten plots were generated for at least three independent batches of PfHsp70-z and PfHsp70-1 (Figure 3.9). The turnover rate of PfHsp70-z reported is comparable to that of PfHsp70-1 (Figure 3.9; Table 3.2); both of which are within the same range as that reported for PfHsp70-1 based on a previous independent study (Matambo et al., 2004; Table 3.2). PfHsp70-z exhibited a lower $K_M$ value (283 μM) compared to that of PfHsp70-1 (428 μM) suggesting that PfHsp70-z has higher affinity for ATP than PfHsp70-1. In
addition, the catalytic efficiency ($K_{cat}/K_M$) of PfHsp70-z was nearly two-fold higher than that of PfHsp70-1 (Table 3.2).

![Figure 3.9: PfHsp70-z exhibits intrinsic ATPase activity](image)

Analysis of the basal ATPase activities of PfHsp70-z and PfHsp70-1 and Pi released was monitored at 595 nm using a direct colorimetric assay.

**Table 3.2: Data for kinetics of the ATPase activities of PfHsp70-z and PfHsp70-1**

<table>
<thead>
<tr>
<th>Protein</th>
<th>$V_{max}$ (nmol/min/mg)</th>
<th>$K_M$ (µM)</th>
<th>$K_{cat}$ (mol⁻¹)</th>
<th>$K_{cat}/K_M$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PfHsp70-z</td>
<td>19.8 (+/- 0.5)</td>
<td>283 (+/- 3)</td>
<td>1.9</td>
<td>6.71 E⁻03</td>
<td>This study</td>
</tr>
<tr>
<td>PfHsp70-1</td>
<td>24.6 (+/- 0.8)</td>
<td>428 (+/- 5)</td>
<td>1.6</td>
<td>3.73 E⁻03</td>
<td>This study</td>
</tr>
<tr>
<td>PfHsp70-1</td>
<td>14.6</td>
<td>616.5</td>
<td>1.03</td>
<td>1.67 E⁻03</td>
<td>Matambo et al., 2004</td>
</tr>
</tbody>
</table>

Table 3.2 legends: Values for the $V_{max}$—the maximum rate of the catalysis reaction; $K_M$—is the substrate concentration at which the reaction rate is at half-maximum; $K_{cat}$—is the maximum number of substrate molecules converted to product per enzyme molecule per second; $K_{cat}/K_M$ is the catalytic efficiency were determined as shown. Standard deviations shown represent at least three independent assessment made using separate protein batches.

**3.3.10 PfHsp70-z forms higher order oligomers**

The purified recombinant PfHsp70-z appeared both as a 100 kDa monomer and a species of approximately 200 kDa based on SDS-PAGE and Western blot analyses (Figure 3.4B). It was speculated that the band representing the species of approximately 200 kDa was that of a detergent resistant PfHsp70-z dimer. To further validate the oligomeric status of PfHsp70-z, SPR analysis was conducted. The SPR raw data (Figure 3.10) were analysed by applying the
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Langmuir kinetic model (Figure 3.10), to generate the $k_a$, $k_d$ and $K_D$ values for the various SPR sensorgrams (Figure 3.10; Table 3.3).

The ability of PfHsp70-z immobilized on the chip surface to interact with PfHsp70-z in solution was analysed. SPR sensorgrams for specific interactions should show concentration dependence response. Based on the SPR analysis, PfHsp70-z (ligand) associated with its analyte form to generate higher order oligomers in a concentration dependent fashion (Figure 3.10A). The concentration dependence of the association response units suggests that the association was specific. Furthermore, BSA used as a control showed no concentration dependent response when BSA was injected over the immobilised PfHsp70-z. In addition, this association occurred in the absence of a nucleotide (Figure 3.10A) and presence of nucleotides ATP (Figure 3.10B) and ADP (Figure 3.10C) inhibited the association by one order of magnitude when compared to absence of (Table 3.3). Taken together, this suggests that the presence of nucleotides ATP/ADP slightly inhibited self-association of PfHsp70-z forming higher order oligomers.

The fact that this association occurs at nanomolar range (Table 3.3) suggests that the interaction is with fairly high affinity and the absence of ATP/ADP promoted the association. Overall, these findings suggest that PfHsp70-z is capable of forming stable higher order oligomers. It is likely that the protein occurs largely in dimers that are partially resistant to detergents that were resolved by SDS-PAGE and Western analyses (Figure 3.4).
Figure 3.10: PfHsp70-z is capable of forming higher order oligomers

The SPR sensorgrams demonstrating concentration dependent association between PfHsp70-z mounted on the chip and PfHsp70-z introduced in solution. The assay was conducted in the absence of nucleotide (A); and repeated in the presence of 5 mM ATP/ADP (B)/(C), respectively. Association was allowed for 2 min, and the dissociation was monitored for 10 min. The black sensorgram represents the Langmuir kinetic model fit, and the green sensorgrams represent the raw data.

Table 3.3. Kinetics data for the oligomerization of PfHsp70-z

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Presence of Nucleotide</th>
<th>$k_a$ (M$^{-1}$s$^{-1}$) (E+03)</th>
<th>$k_d$ (s$^{-1}$) (E-05)</th>
<th>$K_d$ (nM)</th>
<th>$X^2$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PfHsp70-z</td>
<td>-</td>
<td>12.0 (+/-0.3)</td>
<td>9.54 (+/-0.4)</td>
<td>7.97</td>
<td>3.06</td>
<td>This study</td>
</tr>
<tr>
<td>PfHsp70-z</td>
<td>5 mM ADP</td>
<td>6.62 (+/-0.5)</td>
<td>7.60 (+/-0.5)</td>
<td>11.5</td>
<td>2.27</td>
<td>This study</td>
</tr>
<tr>
<td>PfHsp70-z</td>
<td>5 mM ATP</td>
<td>2.84 (+/-0.1)</td>
<td>3.20 (+/-0.1)</td>
<td>11.3</td>
<td>4.78</td>
<td>This study</td>
</tr>
<tr>
<td>hHsp70</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>4.5</td>
<td>ND</td>
<td>Marcion et al., 2014</td>
</tr>
</tbody>
</table>

Table 1 legends: $k_a$ association rate constant, $k_d$ dissociation rate constant, $K_d$ equilibrium constant, $X^2$ correlation for SPR sensorgram fitting to the Langmuir model, ND not determined. Standard deviations of at least three independent protein batches are shown in parenthesis.
3.4 Discussion

The recombinant PfHsp70-z protein purified appeared both as a monomer and dimer (Figure 3.4B). SPR analysis conducted to validate that of PfHsp70-z molecules are capable of self-association (Figure 3.10). It was possible that recombinant PfHsp70-z proteins interacted as substrates and chaperone. This was highly unlikely considering that recombinant PfHsp70-z proteins were natively purified (Section 3.3.4). The formation of PfHsp70-z higher order oligomers of PfHsp70-z is nucleotide-independent (ATP/ADP) and the interaction exhibits fairly high affinity (Figure 3.10; Table 3.3). These findings are in agreement with independent studies that have suggested that canonical Hsp70 and Hsp110 homologues are capable of forming functionally important dimers (Liu and Hendrickson, 2007; Marcion et al., 2014). Hsp70s in dimer form has been reported to preferentially interact with Hsp40 as compared to monomer state (Marcion et al., 2014; Sarbeng et al., 2015). Oligomerization of Hsp70 highlights the importance of dimer formation in the functional cycles of these proteins. It is plausible that oligomerisation regulates PfHsp70-z function.

This study also sought to establish the secondary structure of PfHsp70-z. The protein possesses α-helix and β-sheets estimated to be 50 % - 62 %; and 10 % - 16 %, respectively (Figure 3.6 A), which correlates with the predicted structural constitution (Section 2.3.4). This is in line with reported structural constitution of its yeast Hsp110 homologue, Sse1 (Liu and Hendrickson, 2007). PfHsp70-z also possesses β-turns (8 %) and a fairly significant amount of unordered segments (14 %). As predicted the unordered segments (Figure 2.5) covering the Tedwely motif are important for protein-protein interactions. The intrinsically disordered regions on proteins have been reported to be responsible for protein-protein interactions which are transient but specific interactions (Hsu et al., 2012; Dyson and Wright, 2015). These findings suggest that apart from interacting with Hsp70-1 as a possible NEF, PfHsp70-z possibly interacts with other functional partners through its disordered regions.
Chapter 3

Production of Recombinant PfHsp70-z Protein and Analysis of its Secondary and Tertiary Structures

The current study findings suggest that at least 60 % of PfHsp70-z is heat stable at temperatures up to 80 °C (Figure 3.6D). The resilience of PfHsp70-z to heat stress is consistent with its possible role as a molecular chaperone as the functions of molecular chaperones is particularly important in response to the development of cellular stress. Since heat shock proteins should be able to keep other proteins in a folding competent form (Shonhai et al., 2007), they should themselves be heat stable. This is important as the parasite has been observed to up-regulate some of its heat shock proteins under heat stress (Oakley et al., 2007). Furthermore, this study established that PfHsp70-z is an ATPase (Figure 3.9). The protein demonstrated higher catalytic efficiency than PfHsp70-1 (Table 3.2). It has been reported previously that PfHsp70-z provides cytoprotection to malaria parasites by preventing the aggregation of asparagine repeats rich proteins of P. falciparum (Muralidharan et al., 2012). Taking into account that about 24 % of P. falciparum proteins possess asparagine rich repeats (Singh et al., 2004), it is not surprising that this protein is essential. Hsp110s are largely thought to act as holdases that are capable of holding client proteins in a folding competent manner under thermal stress (Goeckeler et al., 2002). They are thought to subsequently pass their substrates to other chaperones such as canonical Hsp70s that are capable of refolding them. It is for this reason that their ATPase subdomain is thought to be dispensable as the holdase function of Hsp70/Hsp110 chaperones is independent of ATP hydrolysis (Oh et al., 1999; Goeckeler et al., 2002). On the other hand, some studies have suggested that ATP binding may be important for their holdase function, and not necessarily ATP hydrolysis (Shaner et al., 2004; Hrizo et al., 2007). Although it remains to be established whether nucleotide binding influences the independent chaperone activity of PfHsp70-z (section 4.3.4), the current findings suggest that it is capable of hydrolysing ATP.

This study demonstrated that PfHsp70-z is a heat stable molecule that is capable of forming dimers. The optimization of the over-production of functional recombinant PfHsp70-z paves way for further biochemical analysis to establish its functions in the development of malaria parasites.
CHAPTER 4

INVESTIGATION OF THE ASSOCIATION OF PFHSP70-Z WITH PFHSP70-1 AND ASSESSMENT OF ITS CHAPERONE ACTIVITY IN VITRO
Investigation of The Association of PfHsp70-z with PfHsp70-1 and Assessment of its Chaperone Activity in vitro

4.1. Introduction
The current study showed that PfHsp70-z is a thermally stable molecule that possesses ATPase activity typical of canonical Hsp70s (Section 3.3.9). Hsp70s are known to play a role in protein quality control (Oh et al., 1999; Shonhai et al., 2008). PfHsp70-z has been shown to be an essential protein in parasite survival under heat stress (Muralidharan et al., 2012). It is plausible that, the thermal stability of PfHsp70-z during heat stress is linked to its involvement in protein quality control. A quarter of the *P. falciparum* proteome is composed of asparagine repeat rich proteins which have an increased propensity to aggregate under heat stress (Singh et al., 2004b). The heat stability of PfHsp70-z may enable it to deal with increased demand to minimize heat induced protein aggregation without itself aggregating. The current chapter seeks to establish the chaperone function of PfHsp70-z.

Hsp110s are implicated in nucleotide exchange of canonical Hsp70 counterparts (Dragovic et al., 2006). It is important to investigate whether PfHsp70-z could serve as a potential NEF of PfHsp70-1. To function as a NEF, PfHsp70-z must be able to bind and distinguish nucleotides. PfHsp70-z was established to form higher order oligomers (section 3.3.10). The formation of possible heterodimers of PfHsp70-z with PfHsp70-1, may facilitate nucleotide exchange, hence it was important to investigate the ability of PfHsp70-z to associate with PfHsp70-1. Nucleotides are known to regulate nucleotide exchange function of Hsps (Andreason et al., 2008). This study thus partly sought to determine the effect of nucleotides on the possible PfHsp70-z and PfHsp70-1 association. PfHsp70-z’s direct chaperone function has not been demonstrated. Its physical association with PfHsp70-1 is yet to be demonstrated.

The main objectives of this study were to:

i) Determine the nucleotide binding affinity of PfHsp70-z;

ii) Assess the effect of nucleotides on PfHsp70-z structural conformation using limited proteolysis and tryptophan fluorescence;
Investigation of The Association of PfHsp70-z with PfHsp70-1 and Assessment of its Chaperone Activity in vitro

iii) Explore the chaperone function of PfHsp70-z using MDH and luciferase aggregation assays; and

iv) Investigate the direct interaction between PfHsp70-z and PfHsp70-1.
4.2 Experimental Procedures

4.2.1 Materials

Reagents used in this study were purchased from Merck Chemicals (Darmstadt, Germany), Thermo Scientific (Illinois, USA), Zymo Research (USA), Melford (Suffolk, UK), Sigma-Aldrich (USA). The α-PfHsp70-z antibody specific for PfHsp70-z previously described (section 3.2.1; Zininga et al., 2015a) was used. Polyclonal rabbit raised antibodies specific for PfHsp70-1 that were previously described (section 3.2.1; Shonhai et al., 2008) were used. The other reagents and materials used are listed in Appendix (C1).

4.2.2 Investigation of PfHsp70-z nucleotide binding affinity using SPR

The assay was conducted at room temperature (25 °C) using a ProteOn XPR36 (BioRad, USA). Filter sterilised and degassed PBS-Tween (section 3.2.10) was used as running buffer. PfHsp70-1, PfHsp70-z and PfHsp70-1_{NBD} (as ligands) were immobilised on the HTE chip surface. The immobilisation was achieved through the Tris-NTA complex activated with Ni^{2+}, to enable stable binding of polyhistidine-tagged proteins following manufacturer’s protocol (BioRad USA) (Appendix A13; B11; B12). PfHsp70-1 and PfHsp70-z were immobilised at concentrations of 0.5 µg/mL and 1 µg/mL, PfHsp70-1_{NBD} (as ligand) was immobilised at 1 µg/mL. At these concentrations the response units for each immobilised protein achieved were as follows: 187 response units (RU) for PfHsp70-z, 198 for PfHsp70-1 and 180 for PfHsp70-1_{NBD} RU per immobilization surface. As analytes aliquots of ATP/ADP were prepared at final concentration of 1.25 nM, 2.50 nM, 5 nM 10 nM and 20 nM and were injected at 100 µl/min in each horizontal channel. Association was allowed for two minutes and dissociation was monitored for eight minutes. Steady state equilibrium constant data was processed and analysed using ProteOn Manager™ software version 3.1.0.6. by concatenating the responses of all five analyte concentrations.
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Investigation of The Association of PfHsp70-z with PfHsp70-1 and Assessment of its Chaperone Activity in vitro

4.2.3 Investigation of the effects of nucleotides on the conformation of PfHsp70-z by partial proteolysis

Nucleotide dependent conformational alterations of PfHsp70-z were investigated by partial trypsin proteolysis using a previously described method (Raviol et al., 2006) with modifications. Briefly, 0.45 μg/ml recombinant PfHsp70-z was incubated in the absence and presence of 5 mM nucleotide ATP/ADP in buffer A (137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 1.46 mM KH₂PO₄) for 10 minutes at 30 °C. The proteolytic reaction was started by addition of 0.25 ng/ml of trypsin. Samples were taken at 0, 5, 15 and 30 minute time points and added to 4X SDS loading buffer (0.5 M Tris-HCl, pH 6.8, 10 % (v/v) glycerol, 10 % (w/v) SDS, 5 % (v/v) β-mercaptoethanol, 1 % (w/v) Bromophenol Blue) and analysed using SDS-PAGE and stained using the Pierce® silver stain (Appendix A14). Western analysis was used to verify the production of PfHsp70-z using α-His antibodies (1 : 2000). Imaging of the protein bands on the blot was conducted using the ECL kit as per manufacturer’s instructions. Images were captured using ChemiDoc Imaging system (Bio-Rad, USA).

4.2.4 Investigation of the nucleotide dependent conformational changes of PfHsp70-z using tryptophan fluorescence based analysis

As a follow-up to the limited proteolysis study, nucleotide dependent conformational alterations of PfHsp70-z were further investigated by tryptophan fluorescence as previously described (Section 3.2.9; Raviol et al., 2006). Recombinant PfHsp70-z (0.45 μg/ml) was incubated in the absence and presence of 5 mM nucleotide ATP/ADP in assay buffer A (Section 3.2.9) for 10 minutes at 20 °C and time-course was conducted for 65 minutes. Then fluorescent units were measured as previously described (Section 3.2.9). Relative fluorescence were determined as average for at least 7 spectrum scans after subtraction of baseline (buffer with or without nucleotides in the absence of protein).

4.2.5 Investigation of PfHsp70-z chaperone function using luciferase aggregation assay

The chaperone function of recombinant PfHsp70-z was investigated by analysing its ability to prevent aggregation of heat denatured luciferase from Photinus pyralis (Sigma-Aldrich) using...
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Investigation of The Association of PfHsp70-z with PfHsp70-1 and Assessment of its Chaperone Activity in vitro

spectrophotometry. The aggregation suppression assay was conducted as previously described (Xu et al., 2012) with minor modifications. The assay was initiated by adding 0.2 μM luciferase and 0.2 μM recombinant chaperone in assay buffer C (25 mM HEPES-KOH, pH 7.5, 5 mM MgCl₂, 5 mM NaOAc, 50 mM KCl, 5 mM β-mercaptoethanol) heated to 48 °C. Protein aggregation was monitored for 60 minutes at 320 nm using the SpectraMax M3 spectrometer (Molecular Devices, USA). To determine if the chaperone function was dependent on the presence of nucleotides, 5 mM ATP/ADP was added to the reaction mixture 15 minutes after the reaction had started.

4.2.6 Investigation of PfHsp70-z chaperone function using malate dehydrogenase aggregation assay

As a follow-up study, the chaperone function of recombinant PfHsp70-z was further investigated by analysing its ability to prevent aggregation of heat denatured malate dehydrogenase (MDH) from porcine heart (Sigma-Aldrich, USA). The aggregation suppression assay was conducted as previously described (Shonhai et al., 2008; Luthuli et al., 2013; Makumire et al., 2014). Briefly, the assay was initiated by adding 0.2 μM MDH and 0.2 μM recombinant PfHsp70-z in assay buffer B (50 mM Tris-HCl, pH 7.5, 100 mM NaCl) and reaction mix was incubated at 48 °C. Protein aggregation was monitored as previously described (Section 4.2.5).

4.2.7 Investigation of the direct association of PfHsp70-z with PfHsp70-1 slot blot analysis

Purified recombinant PfHsp70-z (25, 50, 100 pM) and controls were spotted onto nitrocellulose membrane using Bio-Dot™SF apparatus connected to a vacuum pump (Sultana and Butterfield, 2008). BSA was used as negative control. The membrane was blocked with 5 % (w/v) fat free milk in TBST (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1 % (v/v) Tween 20) and overlaid with either PfHsp70-1/PfHsp70-1_NBD (0.4 μM) in the presence of 5 mM ATP/ADP and incubated overnight at 4 °C, followed by three washes with TBST for 15 minutes. The nucleotide concentrations were maintained in the subsequent steps of the protocol. Protein was detected using α-PfHsp70-1 antibody (1: 2000) (Shonhai et al., 2008) and α-PfHsp70-z
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(Genscript, USA) as primary antibodies which were detected using secondary HRP-conjugated α-rabbit IgG antibody (1: 4000) (Sigma-Aldrich, USA). Imaging of the protein bands on the blot was conducted as previously described (Section 3.2.4).

4.2.8 Analysis of the interaction between PfHsp70-z and PfHsp70-1 using surface plasmon resonance

The assay was conducted at room temperature (25 °C), filter sterilised and degassed PBS-Tween (Section 3.2.10) was used as running buffer. The immobilisation of ligands was conducted as previously described (Section 3.2.10) with slight modifications. PfHsp70-1, PfHsp70-z (as ligands) were immobilised at concentrations of 0.5 µg/mL and 1 µg/mL, PfHsp70-1_{NBD} (as ligand) was immobilised as 1 µg/mL. At these concentrations 187 response units (RU) for PfHsp70-z, 196 for PfHsp70-1 and 198 for PfHsp70-1_{NBD} RU were achieved per immobilization surface. As analytes aliquots of PfHsp70-z, PfHsp70-1 and PfHsp70-1_{NBD} were prepared at final concentration of 125 nM, 250 nM, 500 nM 1000 nM and 2000 nM and were injected at 50 µl/min in each horizontal channel. Association was allowed for 2 min, dissociation was monitored for 10 min. Data analysis was conducted as previously described (Section 3.2.10).
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Investigation of The Association of PfHsp70-z with PfHsp70-1 and Assessment of its Chaperone Activity in vitro

4.3 Results

4.3.1 Nucleotide equilibrium binding of PfHsp70-z and PfHsp70-1

PfHsp70-z nucleotide binding affinities were determined using SPR analysis (Figure 4.1). At steady state the ATP affinity for the recombinant proteins were analysed by equilibrium analysis (Figure 4.1). The equilibrium analysis was conducted on the SPR sensorgrams at steady state using varying nucleotide (ATP) concentrations as analyte on the following ligands: PfHsp70-z, PfHsp70-1 and PfHsp70-1NBD. BSA was used as a control ligand and no response units were observed. PfHsp70-1 and its truncated form PfHsp70-1NBD obtained ATP binding affinities ($K_D$ values) in the same order of magnitude (Table 4.1). PfHsp70-z exhibited less ATP binding affinity with one order of magnitude when compared to PfHsp70-1 (Table 4.1).

![Figure 4.1: Equilibrium analysis of ATP binding by PfHsp70-z and PfHsp70-1/PfHsp70-1NBD](image)

The data represents the equilibrium analysis of ATP binding constants for all three proteins. The equilibrium constant $K_D$ were obtained for PfHsp70-z, PfHsp70-1 and PfHsp70-1NBD (A). The analysis was conducted by plotting total response units from the association curves of PfHsp70-z (B), PfHsp70-1 (C), and PfHsp70-1NBD (D) against ATP concentration. The standard deviations of at least three independent assays are shown.

Table 4.1: Comparative affinities of PfHsp70-1 and PfHsp70-z for nucleotides at equilibrium binding phase

<table>
<thead>
<tr>
<th>Protein (Ligand)</th>
<th>ATP (analyte)</th>
<th>$K_D$ (µM) [+/- standard deviation]</th>
</tr>
</thead>
<tbody>
<tr>
<td>PfHsp70-z</td>
<td></td>
<td>25.3 (+/- 1.1)</td>
</tr>
<tr>
<td>PfHsp70-1</td>
<td></td>
<td>3.48 (+/- 0.4)</td>
</tr>
<tr>
<td>PfHsp70-1NBD</td>
<td></td>
<td>3.47 (+/- 0.2)</td>
</tr>
</tbody>
</table>

Legend: Standard deviation shown in parenthesis are for at least three independent assays
Investigation of The Association of PfHsp70-z with PfHsp70-1 and Assessment of its Chaperone Activity in vitro

4.3.2 Effect of nucleotides on PfHsp70-z as determined using limited proteolysis

PfHsp70-z was subjected to partial proteolysis in the absence of nucleotide and in presence of ATP/ADP to investigate the effect of the nucleotides on the conformation on the protein. The profiles of fragments that were generated from PfHsp70-z digested in the absence and presence of ADP/ATP were analysed on SDS-PAGE and Western blot (Figure 4.2).

Prior to proteolytic digestion, it was important to validate protein stability, it was observed that the recombinant PfHsp70-z protein was stable represented by a species of 100kDa. This suggests that PfHsp70-z was stable and did not break into smaller fragments prior to incubation with trypsin. The proteolytic digestion after five minutes of incubation generated species of 55 kDa and 60 kDa fragments (Figure 4.2), in all three samples without nucleotides and with ATP/ADP (Figure 4.2). The absence of digestion fragments in the purified PfHsp70-z (Figure 4.2; Section 3.3.4), suggests that the fragments observed were generated during proteolysis with trypsin. Furthermore, there were species of approximately 35 kDa that were
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obtained in the absence of nucleotide (Figure 4.2) and in the presence of ADP (Figure 4.2). This suggests that the ADP bound form of PfHsp70-z may assume a conformation that closely resembles that of the protein when it is not bound to nucleotide.

After 15 minutes incubation in the presence of trypsin, the nucleotide free and ADP bound PfHsp70-z were to a greater degree digested (Figure 4.2) compared to protein in the presence of ATP. After 30 minutes, nucleotide-free and ADP-bound PfHsp70-z was completely digested (Figure 4.2). This further confirms that the ADP bound form of PfHsp70-z may assume a conformation that closely resembles that of the protein when it is not bound to nucleotide. However, ATP-bound PfHsp70-z generated digestion fragments that were unique and part of the protein remained undigested after 30 minutes of exposure to trypsin (Figure 4.2). However, the fragment that remained after 30 minutes could not be detected by Western blot analysis suggesting that it may have lost its N-terminal His-tag. Altogether, the findings suggest that ATP induced PfHsp70-z to assume a compact conformation.

4.3.3 Tryptophan fluorescence analysis

PfHsp70-z possesses two tryptophan residues at positions W436 and W690. Urea sensitivity was used as a control experiment validating sensitivity of protein to change (Section 3.3.8). On the basis of urea sensitivity, further enquiries were made using nucleotide presence as variables. The observed tryptophan fluorescence spectra variations suggested different conformational states of PfHsp70-z in nucleotide-free buffer and in the presence of ADP/ATP (Figure 4.3). The changes observed in the tryptophan-based fluorescence spectrum further confirmed that nucleotide free (Figure 4.3A) and ADP-bound (Figure 4.3A) forms of the protein assumed similar conformational states (Figure 4.3A). On the other hand, the ATP-bound form of the protein assumed a unique conformation (Figure 4.3A) as confirmed by tryptophan fluorescence analysis. PfHsp70-z protein was further incubated for at least 65 minutes in the presence of ATP, followed by samples collected after set time intervals for time-course fluorescence analysis (Figure 4.3B). Tryptophan fluorescence spectral changes were observed with increase in time of incubation in the presence of ATP (Figure 4.3B). This
suggested conformation changes over time, and these may be inferred to reflect hydrolysis of ATP by PfHsp70-z as the tryptophan fluorescence spectra shifted towards that of ADP-bound state over time (Figure 4.3B).

![Figure 4.3: The conformation of PfHsp70-z is regulated by nucleotides](image)

Tryptophan fluorescence signals obtained upon incubating PfHsp70-z in the absence of nucleotide or in the presence of 5 µM ATP/ADP (A). A time-course assessment of ATP on fluorescence intensity of PfHsp70-z emission peak obtained at 345 nm was conducted (B).

### 4.3.4 PfHsp70-z suppresses heat-induced aggregation of luciferase and Malate dehydrogenase

The chaperone activity of recombinant PfHsp70-z was determined by assessing its ability to prevent heat-induced aggregation of luciferase and malate dehydrogenase (MDH). Based on this assay, heat induced protein aggregation results in increased turbidity which is monitored by taking absorbance readings at 320 nm. First, it was important to establish that PfHsp70-z and PfHsp70-1 were heat-stable. The recombinant proteins did not aggregate at 48 °C, this was in line with findings from secondary and tertiary structure data (Sections 3.3.7; 3.3.8). The model substrates luciferase and MDH were observed to aggregate at 48 °C in the absence of chaperone (Appendix B8; B9). Addition of chaperones to the mixture resulted in suppressed heat induced aggregation (Figure 4.4). As a control, a non-chaperone, BSA, was added to the model substrates but it did not suppress the aggregation of MDH and luciferase resulted in increased heat induced aggregation of model substrates. This validated that the
suppression of heat induced aggregation on model substrates was specific for chaperones. The aggregation values were calculated based on the level of aggregation observed for luciferase and MDH without chaperone set as 100% aggregation.

PfHsp70-1 with known chaperone function (Shonhai et al., 2008) was used as a control. In order to determine the effective chaperone/substrate ratio, it was important to titrate amounts of chaperone (Figure 4.4A, B). PfHsp70-z suppressed the aggregation of luciferase and MDH (Figure 4.4A, B) in a concentration dependent fashion. The optimum chaperone activity was observed at a 1:1 chaperone to either luciferase/MDH ratio (Figure 4.4A; B). Furthermore, a comparison of the capabilities of PfHsp70-z and PfHsp70-1 in suppressing heat-induced aggregation of proteins in vitro was conducted. At the same chaperone concentration, PfHsp70-z suppressed aggregation to a greater degree than PfHsp70-1 (Figure 4.4A, B; \( p < 0.005 \)). Based on these findings, PfHsp70-z’s protein aggregation suppression capability is marginally higher when compared with that of PfHsp70-1 (Figure 4.4A, B). This suggests that PfHsp70-z is a chaperone with heat induced aggregation suppression activity.

The effect of nucleotides on the chaperone function was determined by repeating the protein aggregation assays in the presence of ATP or ADP. The chaperone function of PfHsp70-z was not influenced by nucleotide (Figure 4.4C, D). On the other hand, addition of ATP to the assay mix inhibited the protein aggregation suppressive function of PfHsp70-1 as previously reported (Shonhai et al., 2008) (Figure 4.4C, D). However, ADP did not affect the chaperone function of PfHsp70-1. It is known that ADP enhances substrate binding (holdase) function of Hsp70 (Mayer et al., 2000) and therefore this finding is in agreement with this position for both PfHsp70-z and PfHsp70-1.
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Figure 4.4: PfHsp70-z suppresses heat-induced aggregation of luciferase and Malate dehydrogenase
The heat-induced aggregation of luciferase was assessed in vitro at 48 °C. Dose response assessment of the independent capabilities of PfHsp70-z and PfHsp70-1 to suppress heat-induced aggregation of luciferase (A); MDH (B) the assay was repeated in the presence of 5 µM ADP/ATP for luciferase (C) and MDH (D). The activity of PfHsp70-1 and PfHsp70-z in the presence of ATP compared to the nucleotide-free state and ADP-bound state, was statistically significant (p<0.005), respectively. The assay was repeated for luciferase (E) and MDH (F) to assess the activity for the combination of PfHsp70-z and PfHsp70-1, under various conditions: nucleotide-free buffer (NN); presence of ADP/ATP, respectively. Standard deviations obtained from three replicate assays are shown.
Investigation of The Association of PfHsp70-z with PfHsp70-1 and Assessment of its Chaperone Activity in vitro

The combined effect of PfHsp70-1 and PfHsp70-z on aggregation suppression of luciferase or MDH was more efficient (Figure 4.4E, F) than what was observed for the individual chaperones. However, there was two-fold reduction in chaperone efficiency (Figure 4.4E, F) in the presence of ATP. The reduction in chaperone efficiency for the PfHsp70-1 and PfHsp70-z combination in the presence of ATP may be attributed to the specific inhibitory effect of ATP on PfHsp70-1 activity (Figure 4.4 C, D; Shonhai et al., 2008).

4.3.5 Assessment of the interaction of PfHsp70-z with PfHsp70-1 using slot blot technique

The association of PfHsp70-z and PfHsp70-1 was validated using the recombinant forms of the two molecular chaperones. In order to immobilise the recombinant proteins on nitrocellulose membrane the slot blot technique (Sultana and Butterfield, 2008) was used. Prey protein (PfHsp70-z) was immobilized onto the blot. Firstly, it was important to ascertain whether the α-PfHsp70-1 antibodies to be used in the assay were specific and would not cross-react with PfHsp70-z. The blot with immobilised PfHsp70-z protein and controls BSA; (PfHsp70-1; PfHsp70-1_NBD) were used for immunoblotting using α-PfHsp70-1 antibodies (Figure 4.5A). The antibodies detected bands representing both full length PfHsp70-1 and PfHsp70-1_NBD in a concentration dependent fashion (Figure 4.5A; B). Furthermore, α-PfHsp70-1 antibodies did not cross react with BSA immobilised on the membrane (Figure 4.5A; B) or PfHsp70-z (Figure 4.5A; B).

The interaction of PfHsp70-z and PfHsp70-1 was established by passing the prey proteins (PfHsp70-1 and PfHsp70-1_NBD) over the immobilized bait PfHsp70-z protein (Figure 4.5B). This was followed by conducting immunoblotting to detect PfHsp70-1 bound to immobilised PfHsp70-z using α-PfHsp70-1 antibodies (Shonhai et al., 2008). The detected band intensity increased as the amount of PfHsp70-z immobilised on the blot increased (Figure 4.5C). This suggests that PfHsp70-1 was captured on spots where PfHsp70-z was immobilised. Taken together this data suggests that PfHsp70-z interacted with PfHsp70-1 in a concentration dependent manner. The assay was repeated in the presence of nucleotides (ATP/ADP) and it
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appears ADP did not have a significant effect on the interaction (Figure 4.5C). However, ATP appears to enhance the association (Figure 4.5C) of the two proteins.

Densitometric analysis of the immunoblots confirmed the findings that ATP enhances the association between PfHsp70-1 and PfHsp70-z in a dose dependent fashion (Figure 4.5D). The densitometric data suggest that PfHsp70-z interacted with PfHsp70-1\textsubscript{NBD} and full length PfHsp70-1 under all conditions. These findings suggest that the PfHsp70-1\textsubscript{NBD} is the primary site through which PfHsp70-z interact with PfHsp70-1.

Furthermore, the interaction of PfHsp70-z with PfHsp70-1 occurs in the presence of ATP, it is unlikely that the association is based on PfHsp70-1 acting as chaperone-to PfHsp70-z as substrate since PfHsp70-1 releases its substrates in the presence of ATP (Shonhai et al., 2008; Section 4.3.4). It is highly unlikely that PfHsp70-z misfolded during the assay as it was observed that the molecule is thermally stable (sections 3.3.7; 3.3.8), hence it would not
constitute an appropriate substrate of PfHsp70-1. Furthermore, the natively purified PfHsp70-z protein did not elute complexed with *E. coli* Hsp70 (DnaK) (Section 3.3.4). This further highlights that the purified PfHsp70-z protein was fully folded and not prone to the attention of Hsp70 a through chaperone-substrate association.

In addition, the observed interaction between the NBD subdomain of PfHsp70-1 and PfHsp70-z, further suggests that the association is not based on chaperone-substrate interaction. PfHsp70-1<sub>NBD</sub> lacks the substrate binding domain and therefore could not interact as chaperone and substrate with PfHsp70-z, respectively (Zininga et al., 2015b). However, to rule out that PfHsp70-1<sub>NBD</sub> constitutes a substrate for PfHsp70-z, the observed chaperone function of PfHsp70-z is not responsive to nucleotides (Section 4.3.4). Based on these findings, it is highly unlikely that PfHsp70-z interacted with PfHsp70-1<sub>NBD</sub> as a chaperone and substrate association. In addition, during purification, the recombinant PfHsp70-1<sub>NBD</sub> protein was maintained in native form throughout the purification process (Section 3.3.6). Therefore, these findings suggest that PfHsp70-z interacts with PfHsp70-1, possibly by establishing ionic contacts with specific residues located in the NBD subdomain of the latter.

### 4.3.6 Assessment of PfHsp70-z interaction with PfHsp70-1 using SPR

To further validate observed association of PfHsp70-1 with PfHsp70-z, interaction kinetics and affinity analysis were conducted using SPR based on a simple Langmuir fit model (Figure 4.6). Based on SPR sensor grams for specific interactions there should be concentration dependent response units. As a control, BSA was injected and resulted in no increase in response units. The concentration dependent observation made on the response units during association of PfHsp70-z with full length PfHsp70-1 suggested a specific interaction (Figure 4.6A). The assay was repeated in the presence of nucleotides ADP (Figure 4.6B), or ATP (Figure 4.6C) and similar concentration dependent response units increase were observed. The interaction of PfHsp70-z with PfHsp70-1<sub>NBD</sub> was similarly investigated. The array system allowed for analysis to be conducted by reversing ligand and analyte in the absence of nucleotides (Figure 4.6D) or in the presence of nucleotides ADP (Figure 4.6E) and ATP (Figure 4.6F). The observed
sensorgrams suggest the interactions between PfHsp70-z with PfHsp70-1/PfHsp70-1\textsubscript{NBD} as ligands and analytes were specific as evidenced by the concentration dependence of the response units.

![Image of sensorgrams](image_url)

**Figure 4.6: PfHsp70-z directly interacts with PfHsp70-1/PfHsp70-1\textsubscript{NBD}**

SPR sensorgrams for the interaction of PfHsp70-z with PfHsp70-1/PfHsp70-1\textsubscript{NBD}. The interaction between PfHsp70-1 and PfHsp70-z (A); the experiment was repeated in the presence of 5 mM ADP (B); and 5 mM ATP (C). The interaction of PfHsp70-z and PfHsp70-1\textsubscript{NBD} (D); the experiment was repeated in the presence of 5 mM ADP (E); and 5 mM ATP (F).

The SPR sensorgrams were analysed to generate the SPR kinetics data (Table 4.2). The kinetics data for the interactions were observed for the association and the dissociation phases. The association constant and dissociation constant were determined, respectively. The data was also used to determine the equilibrium constant (binding affinity). The binding affinity data show that ATP promoted interaction in nanomolar range compared to micromolar range in the presence of ADP and absence of nucleotide, respectively (Table 4.2). The interaction kinetics data obtained confirmed the initial results obtained in using slot blot that suggested ATP promoted the interaction between PfHsp70-z with PfHsp70-1/PfHsp70-1\textsubscript{NBD} (Figure 4.5).

The immobilisation order was reversed and PfHsp70-z was immobilised as ligand and PfHsp70-1/PfHsp70-1\textsubscript{NBD} injected as the analyte (Table 4.2). The variation observed in the
interaction kinetics data may be attributed to different orientations of protein immobilisation on GLC surface. Taken together, the SPR interaction kinetics data suggests that PfHsp70-z and PfHsp70-1/PfHsp70-1\_NBD interacts with higher affinity in the presence of ATP than in the absence of nucleotide or presence of ADP (\(p<0.005\)). However, in the presence of ATP PfHsp70-1\_NBD appears to have formed more stable complex with PfHsp70-z than the full length protein (Table 4.2).

### Table 4.2. Kinetics and affinities of PfHsp70-z binding by PfHsp70-1 and PfHsp70-1\_NBD as calculated by langmuir model fitting of sensorgrams

<table>
<thead>
<tr>
<th>Ligand</th>
<th>analyte</th>
<th>Nucleotide</th>
<th>(k_a) (Ms(^{-1}))</th>
<th>(k_d) (s(^{-1}))</th>
<th>(K_D) (M)</th>
<th>(\chi^2)</th>
</tr>
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<tr>
<td>PfHsp70-1</td>
<td>PfHsp70-z</td>
<td>ATP</td>
<td>9.97 (+/- 0.5) e+02</td>
<td>2.42 (+/-1.0) e-05</td>
<td>2.41 e-08**</td>
<td>1.86</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ADP</td>
<td>2.98 (+/- 0.2) e+02</td>
<td>3.61 (+/-0.7) e-03</td>
<td>1.21 e-05</td>
<td>2.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>4.95 (+/- 0.9) e-01</td>
<td>2.96 (+/-0.1) e-05</td>
<td>5.98 e-05</td>
<td>3.32</td>
</tr>
<tr>
<td>PfHsp70-z</td>
<td>PfHsp70-1</td>
<td>ATP</td>
<td>6.08 (+/- 0.8) e+04</td>
<td>7.00 (+/-0.6) e-04</td>
<td>1.15 e-08**</td>
<td>5.67</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ADP</td>
<td>6.52 (+/- 0.3) e+04</td>
<td>1.14 (+/-0.04) e-00</td>
<td>1.75 e-05</td>
<td>2.19</td>
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<tr>
<td></td>
<td></td>
<td>-</td>
<td>4.94 (+/- 1.0) e+04</td>
<td>4.01 (+/-0.5) e-00</td>
<td>8.11 e-05</td>
<td>6.77</td>
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<tr>
<td>PfHsp70-z_NBD</td>
<td>PfHsp70-1_NBD</td>
<td>ATP</td>
<td>4.46 (+/-0.17)e+03</td>
<td>9.43 (+/-0.9) e-06</td>
<td>2.12 e-09**</td>
<td>2.65</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ADP</td>
<td>2.63 (+/-0.7) e+03</td>
<td>2.60 (+/-1.2) e-04</td>
<td>9.86 e-08</td>
<td>6.52</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>2.39 (+/-0.9) e+00</td>
<td>3.76 (+/-0.6) e-08</td>
<td>1.57 e-08</td>
<td>2.44</td>
</tr>
<tr>
<td>PfHsp70-1_NBD</td>
<td>PfHsp70-z</td>
<td>ATP</td>
<td>9.33 (+/-0.4) e+04</td>
<td>1.58 (+/-0.7) e-04</td>
<td>1.16 e-09**</td>
<td>4.39</td>
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<td></td>
<td>ADP</td>
<td>5.80 (+/-0.12) e+04</td>
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<td>1.43 e-08</td>
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<td>5.15 (+/-1.5) e-04</td>
<td>1.24 e-08</td>
<td>1.40</td>
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</table>

**Table Legend**: The interaction kinetics, represented by the association rate constant \(k_a\), dissociation rate constants \(k_d\) and equilibrium constant \(K_D\) were determined by SPR analysis alternating the status of PfHsp70-z and PfHsp70-1 as ligand and analyte, respectively. The ligand was the immobilised protein on the GLC chip surface and the analyte was the protein injected at a flow rate of 50 µl/min. Data was analysed by using running buffer with or without nucleotides in the absence of protein analyte as baseline. Data are represented as mean plus/minus standard deviation of at least five different repeated assays. Chi square (\(\chi^2\)) values shows the langmuir curve fitting and statistical analysis was done using one way ANOVA \((p<0.005)**), showing that there were statistically significant differences in affinity dependent on the presence or absence of ATP.

The SPR kinetics data generated after swapping the ligand/analyte show some degree of variation although they maintain the same order of magnitude (Table 4.2). These variations may be caused by different orientations of the immobilised proteins thereby making some interaction sites available and vice versa. The immobilised proteins form a monolayer on the chip surface; thereby the protein surface on the chip surface has limited accessibility to...
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analyte. Despite these variations the SPR kinetics data are comparable for each data set as indicated by the standard deviation and the goodness of fit (Chi²) less than 10 on the Langmuir model.
Chapter 4

Investigation of The Association of PfHsp70-z with PfHsp70-1 and Assessment of its Chaperone Activity in vitro

4.4. Discussion

The current findings present the first direct evidence that PfHsp70-z possesses independent chaperone function. Furthermore, the study provides evidence confirming that PfHsp70-z interacts with PfHsp70-1 in a nucleotide dependent fashion. The findings indicate that this interaction occurs primarily through the N-terminal ATPase domain of PfHsp70-1.

This study noted that PfHsp70-z binds ATP with high affinity (Figure 4.3.1). This was in line with findings that PfHsp70-z hydrolyses ATP (Section 3.3.9). Therefore, the study enquired the effects of binding nucleotides on the structural conformation of PfHsp70-z. Partial proteolysis which has previously been used as a tool for investigating the conformational changes of Hsp70 induced by nucleotide binding and hydrolysis (Liberek et al. 1991, Raviol et al. 2006) was employed. The similarity in the profile of fragments generated by trypsin proteolysis between native and ADP-bound PfHsp70-z (Figure 4.2A), suggests that ADP has no effect on the conformation of PfHsp70-z. The variation in the fragments produced upon digestion of PfHsp70-z in the presence of ATP versus ADP further testifies to the fact that PfHsp70-z assumes a unique conformation in the presence of ATP. ATP-bound PfHsp70-z is more stable from proteolysis compared to nucleotide-free or ADP-bound PfHsp70-z. This would suggest that PfHsp70-z is protected from trypsin cleavage by conformational changes introduced by ATP. This finding was in line with a previous study that Hsp110 assumes a compact conformation in the ATP bound state (Raviol et al., 2006).

PfHsp70-z possesses two tryptophan residues W436 and W690. The validation of the nucleotide induced conformational changes was conducted using tryptophan fluorescence based study. This study confirms that ATP binding causes PfHsp70-z to assume a distinct conformational state (Figure 4.3A). However, in the presence of ADP, PfHsp70-z assumes a slight conformational shift from the nucleotide free-state.
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Investigation of The Association of PfHsp70-z with PfHsp70-1 and Assessment of its Chaperone Activity in vitro

It was demonstrated that PfHsp70-z is a heat-stable molecule (section 3.3.6; Zininga et al., 2015a). The heat stability of PfHsp70-z substantiates its ability to suppress heat induced aggregation of luciferase and MDH thereby providing direct evidence of its functioning as a heat stable chaperone. PfHsp70-z aggregation suppression is not affected by the presence of ADP/ATP (Figures 4.4). These results suggest mechanistic functional differences between PfHsp70-1 and PfHsp70-z. In light of this, PfHsp70-z possibly possesses a rigid linker that makes it a more robust holdase than PfHsp70-1 under fluctuating ATP levels.

The current study suggests PfHsp70-z interacts with PfHsp70-1 in a nucleotide-dependent fashion (Figure 4.5; 4.6). It was further observed that the truncated form of PfHsp70-1 (PfHsp70-1\textsubscript{NBD}) constitutes the minimum subdomain required for interaction with PfHsp70-z. These findings agree with previous reports on human and yeast Hsp110, that the Hsp110:Hsp70 complex is stable with extensive intermolecular contacts encompassing each NBD (residues 1-384 of each molecule; Schuermann et al., 2008). Removal of the SBD from PfHsp70-1 may expose the PfHsp70-1\textsubscript{NBD} to facilitate better interaction with PfHsp70-z. It was going to be interesting to experimentally validate the nucleotide exchange function of PfHsp70-z but technical limitations made it impossible to be conducted in this study.
CHAPTER 5

ANALYSIS OF THE EXPRESSION, CO-LOCALISATION AND INTERACTION OF PFHSP70-Z WITH PFHSP70-1 IN PARASITES MAINTAINED AT THE RED BLOOD STAGE
5.1 Introduction

The development of malaria parasites at the blood stage is associated with periodic fever conditions. These fever conditions are known to induce select heat shock proteins which are required for maintaining proteostasis in the parasite (Banumathy et al., 2003; Mayer and Bukau, 2005; Pallavi et al., 2010). In addition, the up-regulation of some of the heat shock protein genes during fever conditions augments parasite infectivity (Pallavi et al., 2010). Genomic studies conducted in *Plasmodium* species suggest that genes that have common expression profiles tend to have similar cellular roles (Section 2.3.6; Le Roch et al., 2003). Several heat shock proteins, in *P. falciparum*, are up-regulated in clinical malaria parasite isolates (Pallavi et al., 2010). Some of the main heat shock families amongst them PfHsp70-1 and PfHsp90 were reported to overexpress in response to stress (Pallavi et al., 2010). The association of PfHsp90 and PfHsp70-1 complex has been reported (Gitau et al., 2012; Zininga et al., 2015b); it is not known if PfHsp70-z occurs in this complex. Furthermore, PfHsp70-1 has been reported to be heat inducible (Banumathy et al., 2003; Pesce et al., 2008; Gitau et al., 2012). The heat inducibility of PfHsp70-1, suggests that it plays an important role in cytoprotection of the parasite and the subsequent development of malaria. The current study sought to establish the expression and subcellular localization of PfHsp70-z.

Briefly, the specific objectives of the study were to:

i) Investigate the heat inducible expression of PfHsp70-z;

ii) Investigate the sub-cellular localization of PfHsp70-z in intraerythrocytic *P. falciparum*; and

iii) Determine the physical association of PfHsp70-z and PfHsp70-1 by immunoprecipitation and pull-down assays.
5.2 Experimental Procedures

5.2.1 Materials and special reagents
The following materials and specialised reagents were used in this study: RPMI1640 (containing L-Glutamine, HEPES, 0.2 mM hypoxanthine, 0.1 mg/ml neomycin, 10 % (v/v) plasma (Gibco), albumax II (Invitrogen); protein A/G beads (Pierce, Thermo scientific), α-PfHsp70-1 (Shonhai et al., 2008); α-PfHsp70-z antibodies (Zininga et al., 2015a); TRITC-conjugates goat anti-rabbit and DAPI were kind donations from Prof Heinrich Hoppe, (Rhodes University Grahamstown, South Africa). The rest of the reagents used in the study are listed in (Appendix C1).

5.2.2 Investigation of heat-induced expression of PfHsp70-z in Plasmodium falciparum 3D7 cells
P. falciparum 3D7 cells were cultured as previously described (Trager and Jensen, 1976; Gitau et al., 2012; Zininga et al., 2015a; b). Sorbitol synchronized parasites were harvested at the trophozoite stage from two fractions. One fraction consisted of parasites that had been subjected to heat shock at 42 °C for two hours and the other were cultured under normal temperature conditions (37 °C) prior to harvesting. RBCs infected with P. falciparum 3D7 were lysed with lysis buffer (137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 1.46 mM KH₂PO₄, 0.1 % (w/v) saponin) and incubated at 25 °C for 10 minutes. After lysis the parasites were collected by centrifugation at 5000 x g for 10 minutes. This was followed by an extensive wash step using PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 1.46 mM KH₂PO₄, pH 7.4). The parasite lysate was subjected to Western analysis to confirm the expression of PfHsp70-z using α-PfHsp70-z antibodies (ThermoScientific, USA). To validate the specificity of the α-PfHsp70-z antibodies rabbit pre-immunization serum was also used. To further validate heat induced expression, a protein with known heat induced expression, PfHsp70-1 was probed with α-PfHsp70-1 (Pesce et al., 2008). A host cell protein, glycophorin was also probed as a loading control using goat anti-glycophorin (Sigma-Aldrich, USA) and HRP-conjugated anti-goat secondary antibodies (1: 5000) (Sigma-Aldrich, USA). Furthermore, Western blotting analysis was conducted using α-PfHsp70-z antibodies to confirm the specificity of the antibodies by
probing their capability to bind recombinant PfHsp70-z as opposed to recombinant PfHsp70-1. Images were acquired as previously described (Section 3.2.4)

5.2.3 Immunofluorescence assay

Immunofluorescence assays (IFAs) were carried out after fixation of the parasite infected RBCs using 4 % paraformaldehyde and 0.0075 % glutaraldehyde at 37 °C for 30 minutes. The fixed cells were permeabilised with 125 mM glycine PBS followed by washing with 0.1 % triton-100 in PBS. Blocking was done using 3 % BSA/PBS for 1 hour at room temperature. The cells were incubated with rabbit anti-PfHsp70-z antibodies (diluted 1:1000 in blocking solution) or rabbit anti-PfHsp70-1 antibodies (diluted 1:100 in blocking solution) at 4 °C overnight. After several PBS washes, anti-rabbit-TRITC antibodies (1:2000) were allowed to bind at room temperature and in the dark for 2 hours. The cells were washed three times in PBS and with DAPI (10 μg/ml) included in the last wash. Fluorescence images were captured using Olympus DP72 microscope.

5.2.4 Investigation of the direct association of PfHsp70-z with PfHsp70-1 using a Co-immunoprecipitation and pull down assay

P. falciparum 3D7 cells were cultured, harvested and lysed as previously described (see Section 5.2.2). The parasite lysates were resuspended in 500 µl immunoprecipitation lysis buffer (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 % (v/v) Tween-20, and containing 1 mM PMSF). Immunoprecipitation was carried out using Pierce® Protein A/G Magnetic Beads following the manufacturer’s protocol with minor modifications (Pierce, Thermo scientific) (Appendix A15). Briefly, parasite lysates containing approximately 300 µg of total protein were suspended in a protein A/G magnetic resin to which α-PfHsp70-z antibodies had been attached. Binding was allowed to occur for two hours at room temperature (25 °C) with gentle agitation. To investigate the effect of nucleotide on PfHsp70-z binding to PfHsp70-1, the suspension was split into three. The first aliquot was supplemented with 5 mM ATP, the other supplemented with 5 mM ADP and the other no nucleotides were added. Following subsequent washing steps using TBS wash buffer (25 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.05 % Tween 20), the immunoprecipitate was eluted in 150 µl of elution buffer (100 mM glycine,
pH 2.5) and thereafter neutralized using 100 mM Tris pH 7.5. The immunoprecipitate pulldown was analyzed by Western blot using α-PfHsp70-1.

In addition, to the immunoprecipitation assay, the direct association of PfHsp70-z and PfHsp70-1 was investigated using polyhistidine tagged recombinant PfHsp70-z immobilized on HisPur cobalt affinity resin to facilitate pull down of PfHsp70-1 from parasite lysate. Parasites released by saponin Lysis of the erythrocytes were re-suspended in Pierce lysis buffer (Thermo Scientific). The parasite lysate (Prey) was mixed with purified recombinant PfHsp70-z (Bait) immobilised on HisPur Cobalt resin (Thermoscientific) and binding was allowed for 4 hours at 4 °C. As a control beads without immobilised recombinant PfHsp70-z was used. The samples were washed extensively using TBS: Pierce Lysis buffer. The bait-prey proteins were subsequently eluted in 250 µL elution buffer (TBS: Pierce Lysis buffer, 290 mM imidazole). To investigate the effect of nucleotide on PfHsp70-z binding to PfHsp70-1, the suspension was split into three aliquots. One aliquot was adjusted to 5 mM ATP, the other to 5 mM ADP and the other no nucleotides were added. The pull-down eluates were analysed with Western blot using anti-PfHsp70-1 antibodies.
Chapter 5

Analysis of the Expression, Co-Localisation and Interaction of PfHsp70-z with PfHsp70-1 in Parasites Maintained at the Red Blood Stage

5.3 Results

5.3.1 PfHsp70-1 is heat inducible

This study enquired if PfHsp70-z is heat inducible in *P. falciparum* parasites growing at the blood stage. The expression of PfHsp70-z in *P. falciparum* 3D7 cells was analysed by SDS-PAGE (Figure 5.1A). As a control uninfected RBCs were also analysed (Figure 5.1A). The SDS-PAGE analyses was for samples drawn from culture that was maintained at 37 °C for two hours (Figure 5.1A) and the other were from samples drawn from culture heat shocked at 42 °C for two hours before harvesting (Figure 5.1A). The SDS-PAGE did not resolve the proteins as distinct bands which was due to the masking effect of haemoglobin from the lysed red blood cells. To further validate the presence of PfHsp70-z, Western blot analysis using α-PfHsp70-z antibodies was employed (Figure 5.1A). It was important to validate the specificity of the α-PfHsp70-z antibodies. The antibodies did not cross react with human proteins in the uninfected RBC lysates (Figure 5.1A). The antibodies detected PfHsp70-z as a species of approximately 100 kDa in both the lysate from cells cultured at 37 °C and those exposed to 42 °C, (Figure 5.1A), respectively. The densitometric analysis of the Western blot validated up-regulation of PfHsp70-z expression in cells heat shocked at 42 °C compared to protein expressed in parasites maintained at 37 °C (Figure 5.1B) (*P*<0.005). Taken together, this suggests the expression of PfHsp70-z at the erythrocytic stage is heat induced.

To further validate the specificity of the antibodies, serum collected from the rabbit prior to immunisation with PfHsp70-z derived peptide was used to probe for non-specific binding. The pre-immune serum did not cross react with uninfected red blood cells and with the parasite cell lysates (Figure 5.1). In order to validate the specificity of the α-PfHsp70-1 (Section 4.3.5) and α-PfHsp70-z Western blot analysis was conducted (Appendix B10). Western blot analysis of the samples showed that α-PfHsp70-1 antibodies are specific for recombinant PfHsp70-1 protein as they did not cross react with recombinant PfHsp70-z protein. Western analysis using α-PfHsp70-1 to probe for a known heat inducible protein PfHsp70-1 was used as a control (Figure 5.1). A densitometric analysis of the blot confirmed heat inducibility of PfHsp70-z (Figure 5.1 C). It was further enquired if the increase in densitometric analysis was not a loading problem, therefore, a house keeping protein, red blood cell glycophorin C (Pesce
et al., 2008; Gitau et al., 2012) was used. Western analysis was carried out on glycophorin blot as a loading control (Figure 5.1D). Altogether, this validates the findings that PfHsp70-z expression is up-regulated during heat stress.

5.3.2 Localisation of PfHsp70-z

Recombinant PfHsp70-z has been observed to interact with recombinant PfHsp70-1 (Section 4.3.5; 4.3.6). The current study enquired if PfHsp70-z co-localises with PfHsp70-1 in the cytosol. Immunofluorescence experiments were conducted on parasites cultured at 37°C at the trophozoite stage. Despite the poor resolution of the images captured, distinct fluorescence signals were detected suggestive of specific signals. The specificity of the secondary antibodies was validated that they did not bind non-specifically to parasites. The TRITC conjugated secondary antibodies did not cross react with parasite cells evidenced by the absence of a fluorescence signal (Figure 5.2A). PfHsp70-z appear to be cytosol localised...
(Figure 5.2B). This observation correlated with the cellular distribution of PfHsp70-z which was reported by Muralidharan and colleagues (2012), in the parasite cytosol.

Parasite cells were also probed using α-PfHsp70-1 and it was observed that PfHsp70-1 occurs in the cytosol (Figure 5.2C). These findings suggest the cytosolic co-localisation of PfHsp70-z and PfHsp70-1.

5.3.3 Immunoprecipitation and pull down assays
PfHsp70-z and PfHsp70-1 were reported to localize to the cytosol of the parasite (Section 5.3.2; Pesce et al., 2008; Muralidharan et al., 2012). This study sought to confirm the physical association of PfHsp70-z and PfHsp70-1 (Section 4.3.5; 4.3.6) in a cell based context. Immunoprecipitation and pull down studies were conducted using the α-PfHsp70-z antibodies and recombinant PfHsp70-z, respectively. The association of PfHsp70-1 and PfHsp70-z has been observed to be nucleotide sensitive (Section 4.3.5; 4.6). As a result, the study sought to establish whether the interaction of the PfHsp70-z and PfHsp70-1 was nucleotide-sensitive. A co-immunoprecipitation and pull down studies were conducted to
establish the association of PfHsp70-z and PfHsp70-1 from parasite lysate. The α-PfHsp70-z antibodies coupled to protein A/G coupling resin were used for immunoprecipitation. Polyhistidine tagged recombinant PfHsp70-z immobilized on cobalt affinity matrix was used for pull down assay. The co-immunoprecipitation and pull down assays were conducted in the presence of ATP or ADP. The immunoprecipitation assay was analysed under reducing conditions and as such α-PfHsp70-z was reduced to heavy chain and light chains. The heavy chains were detected on the western blot as species of approximately 50 kDa. PfHsp70-1 appeared on the Western blot as a band of approximately 74 kDa (Figure 5.3). These data indicate that PfHsp70-z and PfHsp70-1 may exist in a common complex in P. falciparum.

Figure 5.3: Immunoprecipitation and pull-down demonstrating association of PfHsp70-z and PfHsp70-1
Pulldown assays conducted using antibodies against PfHsp70-z and recombinant PfHsp70-z protein. Prey proteins in parasite cell lysate harvested from cultures growing under normal temperature (37 °C) and heat stress (42 °C) conditions. Prey protein was allowed to bind onto columns on which the antibody (A) or recombinant PfHsp70-1 (B) were immobilized respectively. Binding was allowed to occur in the absence of nucleotide (NN), or presence of 5 µM ADP/ATP, respectively. The eluate was analysed under reducing conditions and subsequently probed by Western blot using anti-PfHsp70-1 antibodies. The representation of the densitometric analyses for data is provided (lower panels, C, D, respectively). Lane C, control sample (conducted on column with beads minus anti-PfHsp70-z or PfHsp70 recombinant protein); cell lysate used in control assay was obtained from culture incubated at 37 °C; lanes 37 °C, and 42 °C, immunoprecipitation conducted using cell lysate obtained from cells cultured at the respective temperatures which was allowed to bind to beads onto which anti-PfHsp70-z (A) or recombinant PfHsp70-1 (B) were immobilized, respectively. Arrows in panel A show bands representing PfHsp70-1 (70 kDa) and Ig heavy chain (Ig), respectively.

In the current study, PfHsp70-1 appeared to interact with PfHsp70-z both in the presence of either ADP or ATP (Figure 5.3). However, it appears that less PfHsp70-1 associated with
PfHsp70-z in the presence of ADP than in the presence of ATP from parasite lysates of cells maintained at 37 °C (Figure 5.3). Furthermore, the immunoprecipitation conducted in the presence of ADP show reduced apparent association of PfHsp70-1 with PfHsp70-z which when adjusted to the level of IgG heavy chain suggest that it was possibly a loading problem (Figure 5.3A). The assay was repeated for the lysate from cells that were subjected to heat shock at 42 °C to establish the effects of heat stress on the association of PfHsp70-z with PfHsp70-1. A previous study showed that PfHsp70-1 (Pesce et al., 2008) and PfHsp70-z (Section 5.3.1) are up-regulated in parasites under heat stress. The immunoblots of the eluates drawn from immunoprecipitation and pull down assays show enhanced pull down of PfHsp70-1 (Figure 5.3). The observations were confirmed by the densitometric analysis of the immunoblots (Figure 5.3 C; D). The findings suggest that PfHsp70-1 and PfHsp70-z maintain their association under heat stress. The assay was repeated in the presence of nucleotides (ATP/ADP). The observations show increased pull down of PfHsp70-1 with PfHsp70-z in the presence of ATP when compared to ADP (Figure 5.3). The densitometric analysis conducted on the immunoblots further affirms that ATP enhanced association of PfHsp70-z with PfHsp70-1 (Figure 5.3). Taken together, these observations suggest that heat stress and ATP promote the interaction of PfHsp70-1 and PfHsp70-z.
Chapter 5

Analysis of the Expression, Co-Localisation and Interaction of PfHsp70-z with PfHsp70-1 in Parasites Maintained at the Red Blood Stage

5.4 Discussion

In light of the possible broad spectrum of functions for PfHsp70-z its importance in the survival of *P. falciparum* cannot be overemphasized. It was observed that PfHsp70-z is induced by heat stress in malaria parasites cultured at the blood stage (Figure 5.1). PfHsp70-z’s up-regulation in response to heat stress suggests a key role for this protein in proteostasis.

The PfHsp70-z protein is a heat stable molecule (Sections 3.3.7; 3.3.8), highlighting it as an essential chaperone important for parasite survival under heat stress. PfHsp70-1 is known to be up-regulated during heat stress (Pesce *et al.*, 2008). The up-regulation of both proteins suggest that these proteins are required for proteostasis during heat stress.

The current findings demonstrated the co-existence of PfHsp70-z and PfHsp70-1 in the parasite cytosol (Figure 5.2). The cytosolic co-localisation of PfHsp70-1 and PfHsp70-z may suggest that both proteins may functionally interact in close proximity facilitating proteostasis. Furthermore, co-immunoprecipitation and pull down assays validated that PfHsp70-z physically associates with PfHsp70-1 (Figure 5.3). The association of PfHsp70-1 with PfHsp70-z, as observed for recombinant forms of the proteins (Sections 4.3.5; 4.3.6), was enhanced by the presence of ATP. Heat stress induces over-expression of PfHsp70-z and PfHsp70-1, suggesting the formation of functional complexes under heat stress. Furthermore, PfHsp70-1 was found to form heat stable complexes with PfHsp70-z that co-immunoprecipitated (Figure 5.3). A pull down assay showed that recombinant PfHsp70-z associated with PfHsp70-1 from the parasite. This suggests that PfHsp70-1 recognised the recombinant PfHsp70-z partner further highlighting the specific interaction of these two proteins despite the different expression hosts.

In support of the current study, further work on investigation of the direct role of PfHsp70-z as PfHsp70-1 NEF would be essential in understanding the cellular role of this pathway in *P. falciparum*. 
CHAPTER 6

CONCLUSIONS AND FUTURE PERSPECTIVES
6. Conclusion and Future Work

The current study provided evidence for the physical association between PfHsp70-z and PfHsp70-1. Although, the association of PfHsp70-z with PfHsp70-1 was mostly inferred based on evidence from the recombinant form through slot blot and SPR techniques, the evidence from immunoprecipitation further affirms that PfHsp70-z functionally interacts with PfHsp70-1. The cell based studies indicate that PfHsp70-z is required for survival during heat stress and that it forms stable complexes with PfHsp70-1. This highlights the importance of PfHsp70-z during fever episodes, but as a participant that interacts with PfHsp70-1 in maintaining proteostasis.

PfHsp70-z has been observed to suppress heat induced aggregation of model substrates (Section 4.3.4). *P. falciparum* encodes about 24 % of proteins possessing asparagine rich repeats which are prone to aggregation under heat stress (Singh et al., 2004b). PfHsp70-z is an essential molecule whose knock-out is lethal (Muralindaran et al., 2012). This highlights that PfHsp70-z’s aggregation suppression function is important for parasite survival. Predictive studies previously conducted on *P. falciparum* suggest that PfHsp70-z is possibly the only cytosolic NEF of PfHsp70-1 (Shonhai et al., 2007; Bartrya et al., 2015). This suggests that the essential role of PfHsp70-z in parasite survival could be attributed to this unique function of the protein, apart from its role as chaperone (Muralindaran et al., 2012).

The interaction between PfHsp70-z and PfHsp70-1 may constitute a bottle neck for protein folding in *P. falciparum* which may be ideal for drug targeting. The main NEFs in other systems as in GrpE (Packschies et al., 1997), and Bag-1 (Sondermann et al., 2001) their cytosolic *P. falciparum* homologues have not been described. It was observed that the two chaperones PfHsp70-1 and PfHsp70-z are both localized in the cytosol of the parasite. Their co-localization in the same cell compartment possibly favours their direct interaction. This concurs with previous reports that cystolic Hsp110 directly interacts with Hsp70 as a NEF (Dragovic et al.,...
Due to technical limitations this study failed to validate directly the nucleotide exchange function of PfHsp70-z on PfHsp70-1.

Sequence alignment data suggested that PfHsp70-z possesses conserved Hsp110 structural motifs both in the NBD and SBD that might be involved in mediating the PfHsp70-z-PfHsp70-1 partnership. Sequence variation observed between PfHsp70-z and its human orthologue could suggest structural variation; hence possible selective targeting of PfHsp70-z. This may possibly make PfHsp70-z a potential antimalarial drug target. Furthermore, PfHsp70-z, PfHsp70-1 and PfHsp90 are expressed at the clinical phase of malaria and their expression is reported to mirror malaria pathology (Pallavi et al., 2010). However, it still remains to be experimentally validated if PfHsp70-z and PfHsp90 directly interact. Chloroquine resistance in *P. falciparum* is mapped to a 50 kb segment of Chromosome 7, which includes the *PfHsp70-z* and *PfHsp90* genes (Su et al., 1997). This suggests that PfHsp70-z apart from its potential interaction with PfHsp90 in assisting client protein maturation, it may influence chloroquine resistance. Therefore, further characterisation of PfHsp70-z towards understanding its broad functions is important.

Hsp110-Hsp70 complexes have been implicated in cell division where they modulate the activity of the molecules kenessin-5 motor and cin8, which are required for spindle elongation in yeast and mammalian cells (Makhnevych et al., 2013). The modulation of spindle elongation by molecular chaperones might be a mechanism in which cell division may be controlled under proteostatic stress. The rapid growth cycles of *P. falciparum* in the host facilitates its survival and may influence its pathogenesis (Gerald et al., 2011). Although it is unknown whether PfHsp70-z modulates spindle elongation in *P. falciparum*, it cannot be ruled out that its roles as chaperone and NEFs could regulate this pathway.

Previously it has been proposed that Hsp70 modulates actin filament growth (Tardieux et al., 1998a; b). Actin is thought to participate in vesicle trafficking facilitating endocytosis (Smythe.
et al., 2008) and the involvement of PfHsp70-1 in actin filament polymerization reinforces the importance of PfHsp70-z its functional partner, in parasite survival. Apart from prevention of aggregation (Muralidharan et al., 2012), PfHsp70-z may possibly interact with PfHsp70-1 in fundamental cell division and parasite invasion cycles.

The findings from this study suggest a possible broad spectrum of functions for PfHsp70-z protein. It is important to conduct further studies to elucidate the role of PfHsp70-z in cell cycle regulation and actin filament polymerisation. It is important that work done in this study be followed up with further elucidation of PfHsp70-z structure-functional features using X-ray crystallisation to resolve the protein’s three dimensional structure.
References


CDC (2014). Centers for Disease Control and Prevention. President's Malaria Initiative Seventh Annual Report, 


dynamics of its cochaperone Sti1 and the transfer of Hsp70 between modules. Nat Commun, 6: 6655.


APPENDIX

SUPPLEMENTARY DATA, GENERAL EXPERIMENTAL PROCEDURES AND LIST OF SPECIALISED REAGENTS
APPENDIX A: GENERAL EXPERIMENTAL PROCEDURES

A1 Extraction of plasmid DNA
Plasmid DNA was extracted using Zyppy™ Plasmid Miniprep Kit according to manufacturer’s protocol.

A2 Restriction digest of Plasmid DNA using enzymes
Plasmid DNA was digested using the desired diagnostic restriction enzymes following the method described below. The reagents were set up as follows: Sterile deionised water (16 μl), 10x restriction buffer (2 μl) and DNA (100-200 ng) 2 μl. The reaction was initiated by addition of two units of restriction enzymes. The restriction was allowed to proceed for 2-3 hours at 37 °C. The reaction was stopped by addition of 4 μl of 10x DNA loading buffer (0.25 %) bromophenolic blue and 30 % glycerol). The product was then analysed by agarose gel electrophoresis as described in (Appendix A. 5).

A3 Agarose gel electrophoresis
To prepare 0.8 % (w/v) agarose gel, the required amount of agarose was completely dissolved in 1x TAE buffer (40 mM, 20 mM acetic acid and 1 mM EDTA) by heating with frequent agitation. The agarose was then cooled to 55ºC prior to addition of ethidium bromide (0.5 μg/ml). The agarose gel was allowed to polymerise for 15-30 minutes at room temperature. The gel was placed in the electrophoresis chamber and covered with 1x TAE buffer. Volume of 4 μl of 10x DNA loading buffer (0.25% bromophenol blue + 30% glycerol) was added to 20 μl of sample followed by loading of the samples into the wells. Electrophoresis was conducted at 100 volts for one hour. The gel was then visualised using UV light (GeneGenius Bioimaging System (Syngene), USA).

A4 Preparation of competent E. coli XL1/JM109 Blue cells
Colony of E. coli XL1/JM109 Blue was inoculated in 5ml 2YT broth (1.6 % (w/v) tryptone, 1.0% (w/v) yeast, 1.5 % (w/v) agar, 0.5 % (w/v) NaCl in deionised water) broth and grown overnight with shaking at 37 °C. The overnight culture was diluted 1:100 into 50 ml 2YT broth and thereafter grown with shaking to early log phase of absorbance 0.3-0.6 measured at 600 nm. The cells were harvested by centrifuging at 5,000 g for 10 minutes at 4 °C. The
cells were kept on ice from this point onwards. The cells were resuspended in 10 ml 0.1 M MgCl$_2$ and left on ice for 30 minutes. The suspension was centrifuged for 10 minutes at 2700 g at 4 °C. The cells were pelleted as before and gently resuspended in 10 ml ice cold 0.1 M CaCl$_2$ and then followed by incubation on ice for 4 hours. Centrifugation was carried out at 2700 g at 4 °C for 10 minutes. The competent cells were aliquoted by adding one volume of sterile 30 % (v/v) glycerol mixing and stored at -80 °C until use.

**A5 Transformation of competent cells**

A volume of 2 μl (equivalent to about 10 ng) of plasmid DNA was added into an aliquot of 100 μl of competent cell. The cells were then incubated on ice for 30 minutes followed by heat shocking at 42 °C for 45 seconds and immediately placed on ice for 10 minutes. Volume of 900 μl of 2YT broth was added and then incubated at 37 °C for one hour with gently agitation. The cells were transferred on 2YT plates containing the desired antibiotics followed by incubation at 37 °C overnight.

**A6 Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE)**

Proteins were treated by boiling in SDS sample buffer (0.25% Coomassie Brilliant blue (R250), 2% SDS, 10 % glycerol (v/v), 100 mM tris, and 1 % β-mercaptoethanol) in a ratio of 4:1 for 5 minutes at 95 °C and resolved using 12 % acrylamide resolving gel prepared as shown below (Table A.1). The gel is then transferred into the electrophoresis tank and electrophoresis buffer (25 mM Tris, pH 8.3 250 mM glycine and 0.1% (w/v) SDS) was added. The boiled samples were loaded in respective wells and prestained protein molecular weight markers (ThermoFisher Scientific, USA) were also loaded. The electrophoresis was performed at 150 volts for one hour using the Bio-Rad Mini protein electrophoresis system (Biorad, U.S.A).

**Table A1 Preparation of SDS-PAGE**

<table>
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<th>Reagent (ml)</th>
<th>5 % Stacking gel</th>
<th>12 % Separating gel</th>
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</thead>
<tbody>
<tr>
<td>30 % Bis/acrylamide</td>
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<tr>
<td>10 % Ammonium persulphate</td>
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<tr>
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<tr>
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</tr>
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<td>0.05</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1.05</td>
<td>1.58</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.03</td>
<td>0.0020</td>
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</table>
A7 Western Blot
Proteins were resolved in 12% acrylamide gel as described above (Appendix A.7). Removal of SDS-PAGE gel from the glass plates after completion of electrophoresis process and cutting off of the stacking gel was done. The Whatman filter papers, gel, two scotchbrite fibre pads and nitrocellulose were emmersed in western transfer paper and left to equilibrate at 8 °C for 30 minutes. Preparation of the gel for transfer was done as follows: filter paper was placed on a scotch brite pad; Gel was placed on the filter paper ensuring no air bubbles are trapped; nitrocellulose was placed over the gel; another filter paper was laid on top of the nitrocellulose, followed by another scotch brite pad. The transfer of the protein on the nitrocellulose membrane was performed by running at 100 volts for one hour. The membrane was removed from the sandwich and rinsed using transfer buffer as well as removal of adhering gel on the nitrocellulose membrane using a cotton swab. The blot was stained with ponceau stain to determine the success of the transfer followed by visualising the band using chemiluminescence. The membrane was blocked in 10 ml of (5 % non-fat milk in TBS) for one hour on a rotary shaker set at 1 rpm. The membrane was washed three times in TBS-Tween for 10 minutes followed by incubation of the membrane with primary antibody for one hour. Unbound primary antibody were removed by washing of the membrane three times using TBS-Tween for 10 minutes each wash. The membrane was incubated with secondary antibody for one hour followed by washing of the membrane three times using TBS-Tween.

A8 Enhanced ChemiLumescent (ECL)
The Thermo Scientific Pierce ECL Western Blotting Substrate is a highly sensitive nonradioactive, enhanced luminol-based chemiluminescent substrate for the detection of horseradish peroxidase (HRP) on immunoblots.
1. Remove blot from the transfer apparatus and block nonspecific sites with Blocking Reagent for 60 minutes at room temperature (RT) with shaking. If desired, block overnight at 2-8 °C without shaking. 2. Remove the Blocking Reagent and add the primary antibody working dilution. Incubate blot for 1 hour at RT with shaking or overnight at 2-8 °C without shaking.
3. Briefly rinse membrane in Wash Buffer two times.
4. Wash membrane by suspending it in Wash Buffer and agitating for ≥ 5 minutes. Replace Wash Buffer at least 4-6 times. Increasing the Wash Buffer volume, the number of washes and wash duration may help minimize background signal.

5. Incubate blot with the HRP-conjugate working dilution for one hour at RT with shaking.

6. Repeat Steps 3 and 4 to remove nonbound HRP-conjugate. Note: Membrane MUST be thoroughly washed after incubation with the HRP-conjugate.

7. Prepare the substrate working solution by mixing equal parts of Detection Reagents 1 and 2. Use 0.125 mL Working Solution per cm² of membrane. Note: For best results prepare working solution immediately before use. The working solution is stable for one hour at RT.

8. Incubate blot with working solution for one minute at RT.

9. Remove blot from working solution and place it in a plastic sheet protector or clear plastic wrap. Use an absorbent tissue to remove excess liquid and to carefully press out any bubbles from between the blot and the membrane protector.

**A9 Determination of protein concentration using Bradford assay**

Protein concentration was determined by Bradford’s method (Bradford, 1976). Bovine serum albumen (BSA) standards were prepared using concentration ranging from 0 to 1 mg/ml in 0.15 M NaCl. Bradfords reagent 200 µl (Sigma Aldrich, USA) was added to 10 µl of protein and the reaction incubated in the dark at room temperature for five minutes. Absorbance was read at 595 nm using (SpectraMax M3, Molecular devices USA). The recombinant protein was similarly treated and the protein concentration determined by extrapolation from the standard curve as indicated in (Appendix B3; Figure B1). The readings were prepared in triplicate and the average obtained.

**A10 Determination of protein concentration using Christoph-Leidig webtool assay**

Protein concentration was determined using the Christoph-leidig webtool ([http://christoph-leidig.de/tprot.html](http://christoph-leidig.de/tprot.html)) using the following formula

\[ A = c \times d \times \epsilon \]  
Equation 1

\[ m = n \times M \]  
Equation 2
where $A$: Absorbance at 280 nm
$c$: concentration (mol/l)
$d$: cuvette length (cm)
$\varepsilon$: extinction coefficient (L/mol x cm)
$m$: mass (g)
$n$: quantity (mol)
$M$: molecular unit (g/mol)

**A11 determination of CD molar residue ellipticity**

The analysis of the CD spectrum was conducted by conversion of ellipticity units from the CD spectrometer to molar residue ellipticity. This was achieved using the following formula

$$[\theta] = \frac{(100 \times \theta)}{CMR \times l} \quad \text{Equation 3}$$

Where $[\theta]$: molar residue ellipticity (deg.cm$^2$.dmol$^{-1}$)

100: constant converting path length in meters

$\theta$: ellipticity (mdeg)

$l$: cuvette path length

$CMR$: mean residue concentration

$$CMR = c \times N \quad \text{Equation 4}$$

Where $c$: Protein concentration (mol)

$N$: number of amino acids on the protein

**A12 BioRad GLC chip activation and regeneration protocol**

The GLC sensor chip was conditioned (Appendix B4), Pre-concentration of ligand (Appendix B5); immobilization of ligand (Appendix B6) and stabilised following the manufacturer’s protocol BioRad bulletin 6414.
BioRad HTE chip activation and regeneration protocol

The HTE sensor chip was conditioned (Appendix B11) activated and immobilization of ligand (Appendix B12) following the manufacturer’s protocol BioRad bulletin 6044.

SDS-PAGE Silver staining

After running the SDS-PAGE the gel is carefully removed from the casting plates then placed into staining trays. The polyacrylamide gel was stained using Pierce® Silver Stain Kit, the protocol was followed as per manufacturer’s instructions as follows

1. Wash gel in ultrapure water for five minutes. Replace the water and wash for another five minutes.
2. Fix gel in 30% ethanol: 10% acetic acid solution for 15 minutes. Replace the solution and fix for another 15 minutes.
3. Wash gel in 10% ethanol solution for 5 minutes. Replace solution and wash for another five minutes.
4. Wash gel in ultrapure water for 5 minutes. Replace water and wash for another five minutes.
5. Prepare Sensitizer Working Solution by mixing 1 part Silver Stain Sensitizer with 500 parts ultrapure water.
6. Incubate gel in Sensitizer Working Solution for exactly one minute, then wash with two changes of ultrapure water for one minute each.
7. Prepare Stain Working Solution by mixing one part Silver Stain Enhancer with 50 parts Silver Stain.
8. Incubate gel in Stain Working Solution for 30 minutes.
10. Prepare 5% acetic acid solution as a Stop Solution.
11. Quickly wash gel with two changes of ultrapure water for 20 seconds each.
13. When the desired band intensity is reached, replace Developer Working Solution with prepared Stop Solution (5% acetic acid). Wash gel briefly, then replace Stop Solution and incubate for 10 minutes.
A15 Immunoprecipitation using Pierce® Protein A/G Magnetic Beads

Immunoprecipitation was conducted using Pierce® Protein A/G Magnetic Beads (ThermoScientific, USA), with minor modifications as follows:

1. Combine the antigen sample with 10 μg of antibody. Adjust the reaction volume to 500 μL with the Cell Lysis Buffer (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 % (v/v) Tween-20, and containing 1 mM PMSF). Incubate the reaction for 2 hours at room temperature or overnight at 4 °C with mixing.

2. Place 25 μL (0.25 mg) of Pierce Protein A/G Magnetic Beads into a 1.5 mL microcentrifuge tube.

3. Add 175 μL of Wash Buffer to the beads and gently vortex to mix.

4. Place the tube into a magnetic stand to collect the beads against the side of the tube. Remove and discard the supernatant.

5. Add 1 mL of Wash Buffer to the tube. Invert the tube several times or gently vortex to mix for 1 minute. Collect beads with magnetic stand. Remove and discard the supernatant.

6. Add the antigen sample/antibody mixture to a 1.5 mL microcentrifuge tube containing pre-washed magnetic beads and incubate at room temperature for 1 hour with mixing.

7. Collect the beads with a magnetic stand and then remove the flow-through and save for analysis.

8. Add 500 μL of Wash Buffer to the tube and gently mix. Collect the beads and discard the supernatant. Repeat wash twice.

9. Add 500 μL of purified water to the tube and gently mix. Collect the beads on a magnetic stand and discard the supernatant.

10. Low-pH Elution: Add 100 μL of Low-pH Elution Buffer to the tube. Incubate the tube at room temperature with mixing for 10 minutes. Magnetically separate the beads and save the supernatant containing target antigen. To neutralize the low pH, add 10 μL of Neutralization Buffer for each 100 μL of eluate.
### APPENDIX B SUPPLEMENTARY DATA

#### B1 Conservation level of Hsp110 homologues and orthologues

**Table B.1 Conservation level of Hsp110 homologues and orthologues**

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<td>31.5 (48.0)</td>
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<tr>
<td>mH</td>
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<td>22.3 (43.9)</td>
<td>22.5 (44.0)</td>
<td>22.3 (43.4)</td>
<td>21.8 (43.4)</td>
<td>23.1 (43.7)</td>
<td>23.8 (43.7)</td>
<td>25.8 (44.8)</td>
<td>26.8 (44.8)</td>
<td>24.6 (43.9)</td>
<td>24.8 (43.9)</td>
<td>24.4 (42.9)</td>
<td>93.2 (96.6)</td>
<td>-</td>
<td>31.5 (48.0)</td>
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<tr>
<td>Sse1</td>
<td>19.4 (37.0)</td>
<td>19.4 (37.0)</td>
<td>19.0 (34.6)</td>
<td>21.4 (37.5)</td>
<td>21.5 (40.7)</td>
<td>22.9 (40.8)</td>
<td>23.2 (40.4)</td>
<td>23.4 (47.5)</td>
<td>31.8 (48.0)</td>
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**Table Legend:** sequence identities and similarities are in parenthesis as percentages of Hsp110 homologues from Pf; PnHsp70-2; Pv, PnHsp110; Pk, PhHsp110; Pc, PchHsp110; Pb, PbpHsp110; Py, PypHsp110; Pcy, PcyHsp110; HH, HSPH1; mH, mHsp105; Sse1; Tb, Tbhsp110; Tc, Tchsp110; Lm, LmHsp110; La, Lahsp110; Lb, Lbhsp110. The accession numbers are indicated in Figure 2.1.
### B2 Predicted PfHsp70-z peptide epitopes

#### Table B2 Predicted PfHsp70-z peptide epitopes

<table>
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<th>No</th>
<th>Start</th>
<th>Antigenic Determinant</th>
<th>Length</th>
<th>Antigenicity/Surface/Hydrophilicity</th>
<th>Coil</th>
<th>Amphipatic</th>
<th>Synthesis</th>
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<td>N</td>
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<td>N</td>
</tr>
</tbody>
</table>

Table legend. An extra "C" (highlighted as green) is added to the C terminus (or N terminus) to facilitate conjugation. Positive charged residues (K, R, H) are in blue. Negative charged residues (D, E) are in red. Synthesis: "N" means this peptide is easy to synthesize. "Y" means it has difficulty to synthesize.

### B.3 Bradford assay standard curve

#### Figure B1 Bradford standard curve for protein concentration determination

Bovine serum albumen (BSA) standards of concentration ranging from 0 to 1 mg/ml were prepared and absorbance was read at 595 nm using (Biorad, U.S.A). The linear equation: \( y = 0.2868x \); \( R^2 = 0.9968 \) was used to calculate the protein concentration. The protein concentration determined using Bradford were confirmed with Christoph-Leidig method Appendix A10.
B4. GLC sensor chip conditioning

The GLC sensor chip was conditioned in horizontal orientation 0.5 % (w/v) SDS, 100 mM HCl, 0.85 % (v/v) Phosphoric acid, 50 mM NaOH, and in vertical orientation 0.5 % (w/v) SDS, 100 mM HCl, 0.85 % (v/v) Phosphoric acid and 50 mM NaOH. These injections were done for all the 6 horizontal (L1-L6) X 6 vertical (A1-A6) channels on the sensor chip.
B5. Ligand pre-concentration

The ligand pre-concentration analysis was conducted to determine the optimum pH for immobilisation using 10 mM Sodium Acetate buffers at pH 4.0 and pH 4.5. This was followed by reconditioning of the chip using 100 mM HCl, 0.5 % (w/v) SDS and 0.85 % (v/v) Phosphoric acid.
B6. Ligand immobilisation

Figure B4. GLC sensor chip ligand immobilisation
The ligand immobilisation on the GLC surface analysis was conducted first the chip was activated with 1-ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride and N-hydroxysulfosuccirimide. After activation the protein 0.5-1 µg/ml in 10 mM Sodium acetate buffer pH4.0 was injected at a flow rate of 30 µL/s with contact time of 30 seconds. The Chip was subsequently deactivated using 1M ethanolamine HCl pH8.5 to block any remaining carboxy groups on the chip surface.
**B7 Phosphate standard curve**

![Phosphate standard curve](image)

**Figure B5 Phosphate standard curve for ATP hydrolysis analysis**

Phosphate standards of concentration ranging from 0 to 300 μM were prepared and absorbance was read at 660 nm using (SpectraMax M3, Molecular Devices, U.S.A). The linear equation: $y = 0.0011x$; $R^2 = 0.9999$ was used to calculate inorganic phosphate released during ATP hydrolysis.

**B8 Thermal stability of chaperone compared to luciferase**

![Thermal stability](image)

**Figure B6. Thermal stability of chaperones compared to luciferase**

Heat stability of the recombinant proteins was assessed by the presence of heat induced aggregates measured by increase in absorbance at 320 nm for luciferase using M3 Spectramax (Molecular Devices). Recombinant proteins PfHsp70-z and PfHsp70-1, substrate luciferase, and BSA as a control. Error bars represent standard deviations obtained from three replicates of each assay.
B9 Thermal stability of chaperone compared to malate dehydrogenase

Figure B7. Thermal stability of chaperones compared to MDH
Heat stability of the recombinant proteins was assessed by the presence of heat induced aggregates measured by increase in absorbance at 360 nm for MDH using M3 Spectramax (Molecular Devices). Recombinant proteins PfHsp70-z and PfHsp70-1, substrate MDH, and BSA as a control. Error bars represent standard deviations obtained from three replicates of each assay.

B10 Antibody specificity determination

Figure B8. Validation for antibody specificity against PfHsp70-1 and PfHsp70-z
SDS-PAGE (12%) and western analysis of the purification of PfHsp70-1. Lane M – Page ruler (Thermo Scientific) in kDa is shown on the left hand side; lane P, S – the pellet and soluble fraction of total E. coli lysate, lane F – flow through, lane W – wash, lane E – PfHsp70-1 eluted using 500 mM imidazole, lane Z - purified PfHsp70-z. The western Blots were probed using α-PfHsp70-1 and α-PfHsp70-z.
Appendix B11 HTE sensor chip conditioning

Figure B9. HTE sensor chip conditioning

The HTE sensor chip conditioning was conducted by 0.5 % (w/v) SDS, 100 mM HCl, 300 mM EDTA pH 8.5, for both Horizontal and vertical orientation. The flow rate was maintained at 30 µl/min.
Appendix B12 Ligand Immobilisation on HTE Chip

The ligand immobilisation on the HTE surface analysis was conducted first the chip was activated with 10 mM NiSO₄, pH 6.0. The Ligand was immobilised at 0.5 and 1 µg/ml at a flow rate of 30 µl/min. The Chip was subsequently stabilised using blank buffer (PBST) injections including regeneration (300 mM EDTA, pH 8.5) injections at 100µl/min.

Figure B10. HTE sensor chip activation and ligand immobilisation
# Appendix C: Specialised reagents

## Table C.1 List of materials and specialised reagents

<table>
<thead>
<tr>
<th>REAGENT</th>
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<tr>
<td>Acetic acid</td>
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</tr>
<tr>
<td>Adenosine triphosphate</td>
<td>Sigma, U.S.A</td>
</tr>
<tr>
<td>Agarose</td>
<td>Whitehead scientific, South Africa</td>
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<tr>
<td>Ammonium molybdate</td>
<td>Merck, Germany</td>
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<tr>
<td>Ammonium persulphate</td>
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<tr>
<td>Ampicillin</td>
<td>Sigma, U.S.A</td>
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<tr>
<td>Bovine serum albumin</td>
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<td>Bromophenol blue</td>
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<td>Diethiothreitol</td>
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<td>DreamTaq master mix</td>
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<td>Ethidium bromide</td>
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<td>Glacial acetic acid</td>
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