Characterisation of a *Plasmodium falciparum* type II Hsp40 chaperone exported to the cytosol of infected erythrocytes

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By

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Abstract

Heat Shock 40 kDa proteins (Hsp40s) partner with heat shock 70 kDa proteins (Hsp70s) in facilitating, among other chaperone activities; correct protein transport, productive protein folding and assembly within the cells; under both normal and stressful conditions. Hsp40 proteins regulate the ATPase activity of Hsp70 through interaction with the J-domain. *Plasmodium falciparum* Hsp70s (PfHsp70s) do not contain a *Plasmodium* export element (PEXEL) sequence although PfHsp70-1 and PfHsp70-3 have been located outside of the parasitophorous vacuole. Studies reveal that a type I *P. falciparum* (PfHsp40) chaperone (PF14_0359) stimulates the rate of ATP hydrolysis of the cytosolic PfHsp70 (PfHsp70-1) and that of human Hsp70A1A. PFE0055c is a PEXEL-bearing type II Hsp40 that is exported into the cytosol of *P. falciparum*-infected erythrocytes; where it potentially interacts with human Hsp70. Studies reveal that PFE0055c associates with structures found in the erythrocyte cytosol termed "J-dots" which are believed to be involved in trafficking parasite-encoded proteins through the erythrocyte cytosol. If *P. falciparum* exports PFE0055c into the host cytosol, it may be proposed that it interacts with human Hsp70, making it a possible drug target. The effect of PFE0055c on the ATPase activity of human Hsp70A1A has not been previously characterised. Central to this study was bioinformatic analysis and biochemical characterisation PFE0055c using an *in vitro* (ATPase assay) approach. Structural domains that classify PFE0055c as a type II Hsp40 were identified with similarity to two other exported type II PfHsp40s. Plasmids encoding the hexahistidine-tagged versions of PFE0055c and human Hsp70A1A were used for the expression and purification of these proteins from *Escherichia coli*. Purification was achieved using nickel affinity chromatography. The urea-denaturing method was used to obtain the purified PFE0055c whilst human Hsp70A1A was purified using the native method. PFE0055c could stimulate the ATPase activity of alfalfa Hsp70, although such was not the case for human Hsp70A1A *in vitro*.

Declaration

I declare that this thesis is my own, unaided work. It is being submitted for the degree of Master of Science of Rhodes University. It has not been submitted before for any degree or examination at any other university.

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Ms Phililе Nompumelelo Maphumulo, February 2013

Dedication

This thesis is dedicated to my parents, T.C. and Z.J. Maphumulo, for providing unflinching selfless support over the past years. I would not have made it without your unfaltering encouragement.

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My help comes from the LORD, the Maker of heaven and earth.

Psalm 121:2 (NIV)

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List of Abbreviations

α	Alpha
β	Beta
°C	Degree Celcius
μ	Micro
μM	Micromolar
A	Absorbance
A_{600}	Absorbance at 600 nm
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
APS	Ammonium persulphate
BiP	Binding protein
BSA	Bovine Serum Albumin
bp	Base pairs
DNA	Deoxyribonucleic Acid
DnaJ	Prokaryotic Hsp40
DnaK	Prokaryotic Hsp70
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tetra-acetic acid
ER	Endoplasmic reticulum
g	gram(s)
GF-Region	Glycine-Phenyalanine rich region
HEPES	N-2-hydroxyethyl-piperrazine-N'-2-ethanesulfonic acid

His_6	Hexahistidine tag
Hop	Hsp70/Hsp70 organising protein
HPD motif	Histidine-Proline-Aspartic acid motif
Hsc70	70 kDa Heat shock cognate protein
Hsp	Heat shock protein
Hsp70	70 kDa Heat shock protein
Hsp40	40 kDa Heat shock protein
HT	Host Targeting Signal
IPTG	Isopropyl-β-D-thiogalactopyranoside
iRBC	Infected Red Blood Cell
kDa	kilo Daltons
m	Milli
M	Molar
MC	Maurer's Cleft
mol	mole(s)
mg	Milligram(s)
ml	Millilitre
mM	Millimolar
n	nano
NaCl	Sodium chloride
NEF	Nucleotide Exchange Factor
P_i	Inorganic phosphate
P. falciparum	*Plasmodium falciparum*
PBS	Phosphate Buffer Saline
PEXEL	Plasmodium Export Element
PfSBP-1	*P. falciparum* Skeleton Binding Protein-1

PMSF	Phenyl Methyl Sulfonyl Fluoride
PV	Parasitophorous Vacuole
RESA	Ring-infected Erythrocyte Surface Antigen
RNA	Ribonucleic Acid
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Docecyl Sulphate-Polyacrylamide Gel Electrophoresis
TBS	Tris-Buffered Saline
TBS-T	Tris-Buffered Saline-Tween 20
TE	Tris-EDTA buffer
TEMED	N,N,N',N',-tetramethylelethylenediamine
Tris	Tris-2-amino-2-hydroxymethyl-1,3-propanol
U	Unit(s)
UV	Ultraviolet
V	Volts
v/v	Volume to volume ratio
w/v	Weight to volume ration
YT	Yeast-Tryptone media

Chapter 1 : Literature Review

1.1 Molecular Chaperones

The internal environment of a cell is crowded with high concentrations of proteins and other metabolites, and so poses a threat to newly synthesised proteins where aggregation and degradation of improperly folded proteins might occur. A class of proteins that aid polypeptides fold into their proper, functional structure are known as molecular chaperones (Ellis, 1987). Chaperones have been reported to have a role in the refolding of misfolded proteins (Ellis, 1987), in the folding and translocation of newly synthesized proteins, in the refolding of denatured proteins (Hartl, 1996; Fink 1999) and in the proteolytic degradation of unstable proteins (Hendrick and Hartl 1993). Molecular chaperones recognise and bind to hydrophobic regions of proteins, thereby facilitating the proper folding of proteins to their native tertiary structure (Hendrick and Hartl 1993; Zhu et al., 1996), as well as preventing protein aggregation (Young et al., 2004; Bukau et al., 2006). Chaperones are also necessary for continued maintenance of folded proteins by reducing unfavourable inter-molecular interactions within the cell (Fink 1999). Chaperones are known to protect cells against stressful conditions such as heat shock, exposure to toxins and viral infections (Mayer and Bukau, 1998). In addition, molecular chaperones facilitate localisation, import and/or export of target protein and the degradation of aggregated proteins (Feder and Hofmann, 1999).

1.2 Heat Shock Proteins

Heat shock proteins (Hsps) represent an abundant group of proteins whose expression is up-regulated in the event of physical stress, whilst heat shock cognate proteins (Hscs) are constitutively expressed in the cell. Heat shock proteins function by assisting in the refolding of misfolded proteins that have lost native structure to stressful conditions, thereby restoring homeostasis in the cell (Hendrick and Hartl 1995). Hsps constitute the biggest family of molecular chaperones, although not all chaperones are Hsps (Hendrick and Hartl, 1993). Hsps

are classified according to their molecular mass in kilodaltons (kDa) (Zhu et al., 1996). Heat shock proteins are named according to their molecular sizes. The 40kDa heat shock protein (Hsp40), 70 kDa heat shock protein (Hsp70) and the 90 kDa heat shock protein (Hsp90) are amongst those that have been characterised.

1.2.1 Hps70 proteins

Hsp70 is one of the major classes of heat shock protein families. Hsp70s (termed DnaK in prokaryotic organisms) can be expressed in response to cellular stress (inducible Hsp70) but they are also found constitutively expressed in cells (Bukau and Horwick 1998). Hsp70s have been reported to bind to peptide substrates and allowing the proteins to refold (Szabo et al., 1994). Featured across almost all organisms, Hsp70s are found in various compartments of eukaryotic cells including the cytosol, the nucleus, the mitochondria and the lumen of the endoplasmic reticulum (ER) (Hendrick and Hartyl, 1993; Johnson and Craig, 1997). Hsp70s have been reported of having cytoprotective functions towards the malaria parasite in the human host (Sharma, 1992) and are involved in cellular processes such as protein translocation (Gambill et al., 1993; reviewed by Jensen and Johnson 1999), DNA replication (Song et al., 2005), signal transduction (Asea et al., 2002), assembly and disassembly of multiprotein complexes (Song et al., 2005) and protein degradation (Bercovich et al., 1997). In order to optimally carry out their function, hsp70 proteins rely on an ATPase-controlled cycle involving the hydrolysis of ATP to ADP (Powers et al., 2010).

Structural Features of Hsp70s

Hsp70 proteins feature two distinct domains with a total molecular mass of about 70 kDa: These are the 45 kDa domain at the N-terminus that binds ATP (ATPase domain), connected via a linker to the 25 kDa peptide substrate binding domain located at the C-terminus (Figure 1.1).

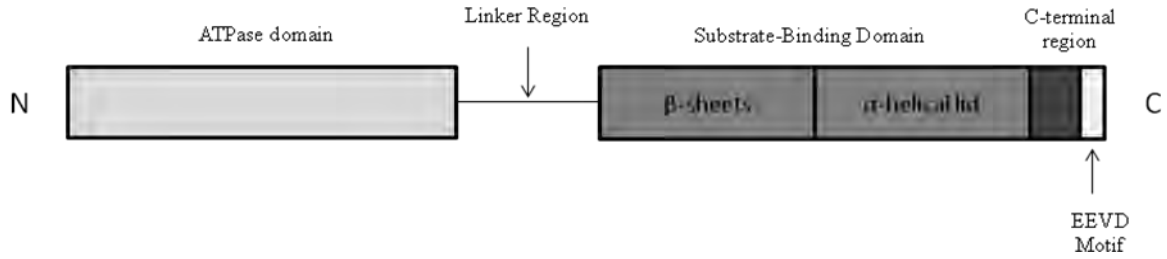

Figure 1.1: Schematic representation of Hsp70 domain organisation.
The Figure shows the N-ternimal ATPase domain linked to the substrate-binding domain via a linker region. The C-terminal substrate-binding domain is further subdivided into β-sheets and the α-helical lid segments. The extreme end of the C-terminus contains the EEVD motif.

The ATPase Domain

The N-terminal nucleotide binding domain (NBD) has ATPase activity and comprises four sub-domains, featuring two lobes, and an upper and lower cleft (Flaherty et al., 1990, 1991; Bork et al., 1992). The upper cleft is further divided into two sub-domains, IB and IIB, whilst the lower cleft is constituted by sub-domains IA and IIA (Flaherty et al., 1990; Powers et al., 2010). Certain highly conserved residues within the ATPase domain, including lysine 71, threonine 199 and aspartic acid 10, are essential for ATP hydrolysis (McCarty and Walker, 1991; O'Bien et al., 1996). The substrate binding capabilities of the substrate binding domain are known to be regulated by conformational changes in the upper cleft, which are induced by the hydrolysis of ATP to ADP (Buchberger et al 1995; Fung et al., 1996). The communication between the ATPase and substrate binding domains is thought to be made possible by the linker region (Jiang et al., 2005). The ATP-bound state of Hsp70 results in a low affinity for the peptide substrate, which is increased in the ADP-bound state (Suh et al., 1999).

The substrate Binding Domain

The substrate binding domain (SBD) of Hsp70 features a hydrophobic peptide-binding sub-domain which is made up of a sandwich of β-sheets and a flexible α-helical lid-like structure (Zhu et al., 1996; Powers et al., 2010). The client polypeptide binds in the substrate-binding

site formed by two loops extending from the β-sandwich (Powers et al., 2010). The substrate-binding site is covered by the α-helical lid which opens upon substrate binding and release. Not only has the lid been suggested to mediate substrate specificity (Mayer et al., 2000), but it has been noted that it also plays a role in stabilising the β-sandwich (Moro et al., 2004). The interaction between the two sub-domains has been suggested to be necessary for substrate entry into the peptide binding pocket (Fernandes-Saiz et al 2006) as it mediates the opening (in the ATP-bound state) and closing (in the ADP-bound state) of the Hsp70 protein (Fernandes-Saiz et al., 2006; Mayer et al., 2000). Recent studies reveal that interaction of DnaK with DnaJ is through the substrate-binding cleft of the SBD (Ahmad et al., 2011).

The C-terminal Domain

The C-terminal region is known to facilitate interactions with other chaperone proteins such as the eukaryotic cytosolic Hsp70 protein (Demand et al., 1998), however, much about its structure is yet to be resolved. The extreme end of the C-terminus contains the EEVD motif. The EEVD motif has a role in Hop binding. Hop (Hsp70/Hsp90 organising protein) is a co-chaperone that provides the link between Hsp70 and Hsp90 and targets Hsp90 to Hsp70 (Chen and Smith, 1998). Hop contains three tetratricopeptide repeat domains (TPR) that have binding sites for the conserved EEVD C-terminus of Hsp70 and Hsp90 (Brodsky et al., 2006). Hsp70 and Hsp90 both bind Hop, which co-ordinates Hsp actions in folding protein substrates. The binding ability of Hop has been localized to the C-terminus of Hsp70 (Demand et al., 1998). However, truncation of the EEVD motif was unable to disrupt Hop binding (Carrigan et al., 2004).

1.2.2 Hsp40 proteins

Hsp40s contain a signature J-domain of a 70 amino acid region which is similar to the 73 amino acids of the *E. coli* DnaJ protein, through which interaction with partner Hsp70 proteins is made possible (Kelley, 1998; Feldman and Frydman, 2000). Hsp40s regulate the ATPase activity of Hsp70s by interacting with the ATPase domains (Cheetham et al., 1994; Kelley, 1998; Kampinga and Craig, 2010). Hsp40s are classified into four types, depending on the number and type of domains they contain (Figure 1.2). Type I Hsp40s have the J-domain, a Gly-Phe-rich (G/F) region, four cysteine-repeats that form a zinc-like domain and a C-terminal substrate binding domain (SBD) that is largely uncharacterised. Type II Hsp40s have the J-domain and the G/F region and the SBD, but lack the cysteine-repeat region. Type I and type II Hsp40s are thought to interact with Hsp70 and facilitate the process of protein folding (Kelley, 1998; Walsh et al., 2004), although the type I and type II subclasses are structurally and functionally diverse (reviewed by Kampinga and Craig, 2010). Type I Hsp40 proteins have been shown to bind to non-native peptide substrates, thus preventing their aggregation (Chae et al., 2004; Lu and Cyr, 1998). Type II Hsp40s have been shown to bind peptide substrate but cannot suppress protein aggregation in the absence of Hsp70 (Chae et al., 2004; Fan et al., 2003; Freeman and Morimoto, 1996; Lu and Cyr, 1998). The J-domain is the only region that is common between the type I, type II and type III Hsp40s. A recently identified fourth class of Hsp40s are the type IV Hsp40s in which the invariant His-Pro-Asp (HPD) is not conserved (Botha et al., 2007).

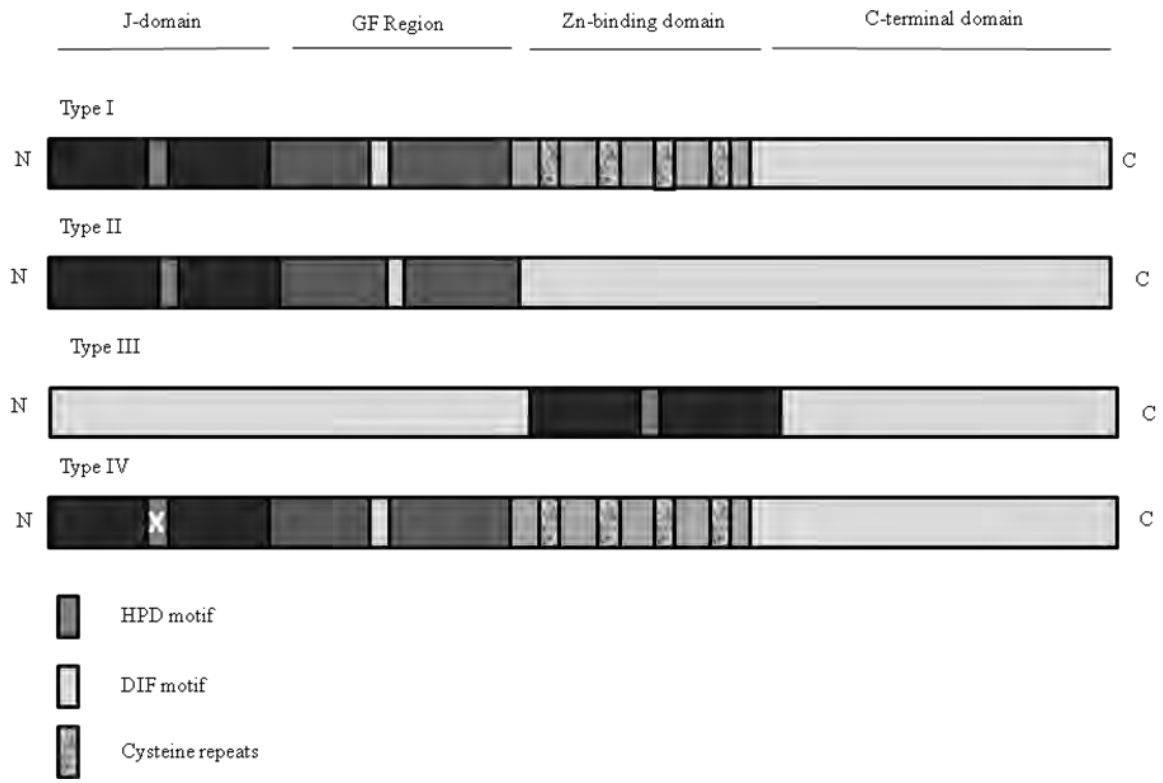

Figure 1.2: Schematic representation of proteins of the Hsp40 family characterised by the presence of four domains.
The Figure shows four types (type I-IV) of Hsp40 proteins that have been classified according to the domain organisation. The sub-domains shown are the J-domain, the GF-rich region, the zinc finger region and the C-terminal domain.

The J-Domain

The J-domain is a highly conserved domain which is believed to be the basic requirement to make interaction between Hsp40 and Hsp70 proteins possible (Corsi and Schekeman 1997; Landry 2003; Mayer et al., 1999). This domain comprises four α-helices (I – IV) (Figure 1.3) that are stabilised by an internal core of hydrophobic residues. Helix I is conserved in type I Hsp40s, but such is not the case for type II and type III Hsp40s (Hennessy et al., 2000). The fairly conserved hydrophobic residues that have been identified in helix I are proposed to be important for the J-domain to maintain its function (Hennessy et al., 2005b; Nicoll et al., 2007). Located between the anti-parallel helices II and III, is a solvent-exposed loop region where the highly conserved HPD motif is located (Qian et al., 1996). This HPD tripeptide is essential for the stimulation of the Hsp70 ATPase activity by Hsp40 proteins. Its alteration

resulted in loss of both J-domain function and Hsp70-interaction in both prokaryotic and eukaryotic cells (Tsai and Douglas 1996; Laufen et al., 1999; Mayer et al., 1999; Hennessy et al., 2005b).

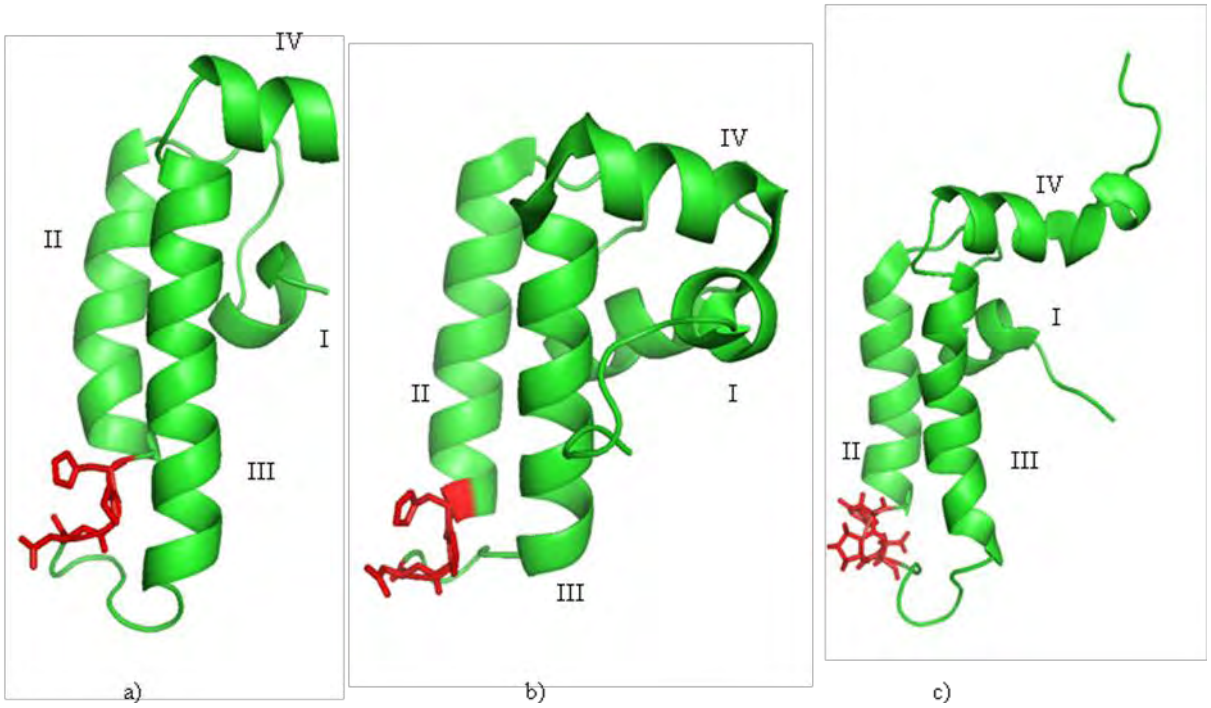

Figure 1.3: Cartoon representations of homology models of J domains PFE0055c, DnaJ and Sis1.
The models (a: PFE0055c; b: DnaJ and c: Sis1) were generated using SWISSMODEL. The HPD motifs are indicated by red sticks. The four helices are marked I to IV. The structures were visualised using PyMOL. The J-domain consists of four α-helices (figure 1.3). The highly conserved HPD motif was observed in the loop region separating helices II and III (red sticks on Figure 1.3) and it's obstruction has been shown cause loss of J-domain function (Hennessy et al., 2005b).

Furthermore, a recent study excluded the HPD motif from being involved in interacting with DnaK in the ADP-bound state (Ahmad et al., 2011). Helix II exhibits other residues which have also been implicated in the interaction between Hsp40 and Hsp70 proteins (Hennessy et al., 2005a). Hennessy et al (2005a) proposed that the phenylalanine residue of a highly conserved KFK motif in helix III may interact with the histidine residue of the HPD motif in the loop region, thereby stabilising the latter residue. Residues of helix IV have been implicated in key structural or functional roles in other Hsp40 homologues (Garimella et al., 2006; Hennessy et al., 2005b). A pentapeptide motif, QKRAA, has been implicated in

enhancing the specificity of J-domain interactions with Hsp70 proteins (Hennessy et al., 2005b). Studies have shown that the N-terminal J-domain, as well as the adjacent G/F-rich region of DnaJ; are required for the interaction with DnaK (Karzai and McMacken 1996). However, it has been revealed that the J-domain alone is sufficient to stimulate the ATPase activity of Hsp70 protein (Wall et al., 1994) through electrostatic interactions with the Hsp70 ATPase domain (Greene et al., 1998).

The Gly/Phe-rich Region

While the G/F-rich region of Hsp40 proteins may not be crucial for the stimulation of Hsp70 proteins (Chamberlain and Burgoyne, 1997), it is believed to have a role in the modulation of DnaK interaction with substrate proteins (Wall et al., 1995). Cajo et al., (2006) established by domain swapping experiments, that the G/F region is essential for the complete *in vivo* function of DnaJ and hypothesised that the DIF (aspartic acid, isoleucine/valine, phenylalanine) motif, a highly conserved tripeptide within the G/F-rich region, plays a role in the hydrolysis of ATP by DnaK, although not directly involved in the stimulation of its ATPase activity (Chamberlain and Burgoyne, 1997).

The Zinc Binding Domain

The zinc finger region is a cysteine-repeat subdomain that contains four repeats of the consensus sequence CysXXCysXGlyXGly where X represents any amino acid. This region forms two zinc fingers which may form a pocket with a hydrophobic core (Banecki et al., 1996; Szabo et al., 1996). Four cysteine-repeat sequences coordinate the binding of a Zinc ion (Cheetham and Caplan 1998). The deletion of the cysteine-rich region in DnaJ does not affect the affinity of DnaJ for DnaK *in vitro*, although it does reduce the stability of the interaction between DnaJ and DnaK (Banecki et al., 1996). This suggests that this region plays a role in the stabilization of the tertiary structure of Hsp40 proteins (Greene et al., 1998; Martines-

Yamout et al., 2000). The mutated DnaJ also had reduced affinity for some of its peptide substrates (Banecki et al., 1996), suggesting that the presence of the zinc binding domain and the C-terminal domain are the minimum requirement for substrate binding (Han and Christen, 2003; Shi et al., 2005; Szabo et al., 1996).

The C-terminal Domain

The C-terminal domain is not as well conserved as the first three regions of Hsp40 proteins (Banecki et al., 1996) and is involved in substrate selection and transfer to Hsp70 (Minimani et al., 1996). This region has been implicated in substrate binding (Szabo et al., 1996), and is the major determinant of substrate specificity for type I and type II Hsp40s (Greene et al., 1998; Kampinga and Craig 2010). Removal of the C-terminal domain from Hsp40 compromises its function (Johnson and Craig, 2001; Sha et al., 2000), highlighting its involvement in dimerization (Borges et al., 2005; Sha et al., 2000; Shi et al., 2005).

1.3 Interactions between Hsp40s and Hsp70s

Hsp40s serve as co-chaperones to Hsp70s; regulating the intrinsic ATPase activity of Hsp70 (Figure 1.4). Activity regulation includes the targeting of substrate to Hsp70, as well as the stabilisation of substrate-bound Hsp70 in the presence of ATP (1) (Fan et al., 2003; Laufen et al., 1999; Suh et al., 1999). Hsp40 co-chaperones regulate ATP hydrolysis by Hsp70 chaperones, allowing them to adopt an ADP-bound conformation with increased affinity for substrate (2) (Flynn et al., 1989; Greene et al., 1998., Suh et al., 1998). Client peptides exhibit hydrophobic residues by which they are recognised by both Hsp70 and Hsp40 chaperones (Takenaka et al., 1995; Li and Sha, 2004). Bacterial DnaK and DnaJ can bind the same substrates although they may have differences in the recognition motifs (Rüdiger et al., 2001) and both chaperones have been shown to interact with the same substrate through different sites (Kim et al., 2002). The Hsp40 protein is thought to bind non-native proteins first, and

then present them to Hsp70 (Hartl 1996; Rüdiger et al., 2001; Walsh et al., 2004). This role has specifically been shown to be fulfilled by type I and type II Hsp40s (Walsh et al., 2004) because they are able to modulate chaperone activity of Hsp70 proteins as well as bind substrate (Walsh et al., 2004). Generally, there are several Hsp40s in cells and a small group of Hsp70s, hence several Hsp40s could interact with one Hsp70 (Cyr, 1995; Walsh et al., 2004). Therefore a diverse range of functional partnerships occurs between few Hsp70s and several Hsp40s (Cyr, 1995; Walsh et al., 2004).

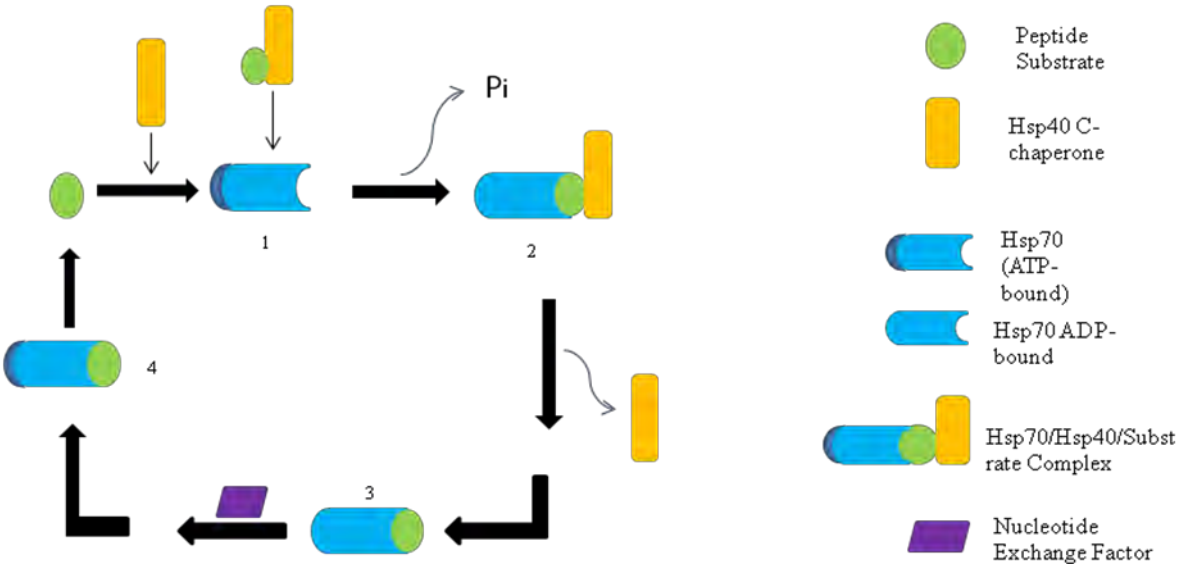

Figure 1.4: The Hsp70 chaperone cycle.
The Figure shows repeat cycles of substrate binding and release, which characterise Hsp70 folding activity. 1) substrate is recognised independently by Hsp70 or delivered to ATP-bound Hsp70 by the co-chaperone Hsp40. 2) Hsp40 interacts with Hsp70 and promotes the hydrolysis of ATP to ADP, increasing substrate affinity leading to 3) folded substrate. 4) Nucleotide exchange factor (NEF) facilitates nucleotide exchange and the folded substrate is released.

ATP controls the Hsp70/Hsp40 chaperone-mediated folding cycle (Mayer et al., 2000). When bound to ATP, Hsp70 has low affinity for substrate peptides, leading to release of substrate (Liberek et al., 1991). ATP hydrolysis locks the substrate in place (Liberek et al., 1991;

Stirling et al., 2003) and the Hsp70 affinity for the substrate is enhanced in its ADP-bound form (Liberek et al., 1991), due to a change in conformation that results in the stabilization of HSP70-polypeptide complexes (Fan et al., 2003). This gives the bound protein the opportunity to refold (3). The substrate is released when ADP is exchanged for ATP by interaction with the nucleotide exchange factors (4) such as GrpE of BAG1 in prokaryotes and eukaryotes respectively, reversing the conformational shift. The J-domain of Hsp40 binds to a cleft in the ATPase domain of Hsp70, leading to significant stimulation of the otherwise low basal ATPase activity of Hsp70 (Hennessy et al., 2005b). The presence of peptides bound in the polypeptide binding site enhances the ability of the J-domain to stimulate Hsp70 ATPase activity (Hennessy et al., 2005b). The lower substrate-binding affinity exhibited by Hsp40s may encourage the transfer of substrate to Hsp70 (Rüdiger et al., 2001).

1.4 Heat Shock Proteins in Plants

Plants suffer from factors which can cause damage to the plant and lead to stresses which are primarily drought, salinity, chemicals, and hot and cold temperatures. Biotic factors, such as herbivores and pathogens, also put plants under stress. Such stresses induce the production of heat shock proteins. Secondary (osmotic and oxidative) stresses basic stresses (drought, salinity, temperature and chemical pollutants) simultaneously acting on plants (Wang et al., 2003). Gene expression and synthesis of heat shock proteins in cells that are exposed to stress are induced by almost all kinds of stresses (Feige et al., 1996). In plants, Hsp70 functions as a molecular chaperone and plays a role in protecting the cells from the effects of heat stress (Choi and Choi, 2009). Studies indicate that Hsp70 found in the stroma of chloroplasts play a role in photo protection (Schroda et al., 1999). A more recent study on *Arabidopsis thaliana* (*A. thaliana*) reveals the necessity of Hsp70 in plant development, and in the thermotolerance of germination seeds (Su and Li, 2008). The overall genome content of *P. falciparum* was found to be more similar to that of *A. thaliana*, when compared to other taxa (Gardner et al.,

2002). Recently, a highly active Hsp70 was identified from the alfalfa plant (*Medicago sativa*) (He et.al., 2008). The alfalfa Hsp70 contained all the conserved domains found in Hsp70 isoforms form different species. Biochemical assays indicated that alfalfa Hsp70 had a high ATPase activity, meaning that it may be useful as a positive control for biochemical assays (Cockburn et.al. , 2011).

1.5 Heat Shock Proteins in Malaria parasite

1.5.1 Malaria

Nearly two hundred and sixteen million malaria cases were reported in 2010 (WHO, 2011), leading to over 655 000 deaths in 2011 (WHO, 2011). Malaria illness in humans is a result of infection with a eukaryotic protozoan parasite, belonging to the *Plasmodium* genus. *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale* and *Plasmodium knowlesi* are all species known to cause malaria in humans. *Plasmodium falciparum* (*P. falciparum*) causes cerebral malaria, the most lethal form of malaria. Eighty one countries worldwide have been identified as *P. falciparum* endemic countries (WHO, 2011), thus, *P. falciparum* remains the most lethal form of malaria.

1.5.2 The life cycle of *Plasmodium falciparum*

The *P.falciparum* life cycle transcends across two habitats (Figure 1.5), the poikilothermic mosquito vector and the warm-blooded human host. When an infected female of the *Anopheles* genus takes a blood meal, it injects malaria sporozoite-containing saliva into the victim's bloodstream (1). The sporozoites then rapidly move into the hepatocytes where they undergo asexual reproduction (2). Upon release from the liver cells (3), the subsequent merozoites initiate the blood stage of the infection by invading erythrocytes (4). Once in the red blood cell (RBC) the merozoites enlarge, maturing into trophozoites (5) and finally forming schizonts. Mature schizonts give rise to more merozoites which are released upon

rupture of the infected RBC (iRBC) (6) and invade new erythrocytes (7). A portion of the parasites differentiate into sexual forms which are macrogametocytes (female) and microgametocytes (male). Macrogametocyte and microgametocyte-containing erythrocytes are taken into the midgut of the *Anopheles* mosquito upon feeding (8), where the mature macrogametocytes escape from the erythrocyte and form macrogametes. Microgametocytes each form eight haploid motile microgametes which quickly move to fertilize a macrogamete, forming a zygote (9), which develops into a motile ookinete (10). The ookinete transverses the midgut epithelium and reaches the extracellular space between the midgut epithelium and the overlaying basal lamina. It then matures into an oocyst, which replicates rapidly to produce sporozoites. Thousands of sporozoites are released (11) into the haemacoel 10 – 24 days after fertilization, invading the mosquito salivary gland epithelium. The cycle is repeated when the mosquito feeds again.

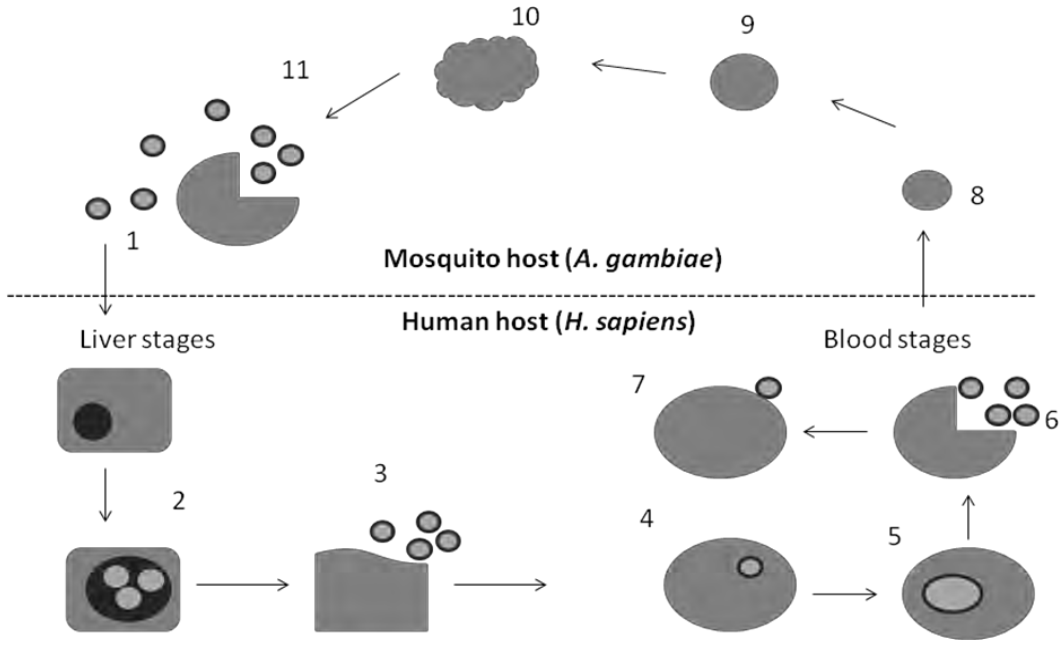

Figure 1.5: The life cycle of malaria parasites
Stages of the development of malaria in the human and mosquito host. Details of the individual stages are provided in the text.

1.5.3 Erythrocyte remodelling

The mature human erythrocyte lacks most organelles of a typical eukaryotic cell, thus to ensure survival, the parasite needs to employ its own intracellular systems, conducive to its growth and development. The remodelling of the infected erythrocyte must allow nutrient acquisition, protein trafficking, protein degradation, replication and evasion of the host immune response (Przyborski and Lanzer, 2005). The remodelling is made possible by the export of proteins of parasite origin (Haldar et al., 2005; Lingelbach and Przyborski, 2006; Templon and Deitsch, 2005). The exported proteins, collectively known as the exportome or secretome, (Hiller et al., 2004; Marti et al., 2004, Sergeant et al., 2006) have many different functions in the infected erythrocyte. Following export of hundreds of proteins into the erythrocyte, the structural and functional properties of the cell are modified, resulting in increased rigidity and cytoadherence (Maier et al., 2008). During parasite invasion of the erythrocyte, the parasitophorous vacuole (PV) (in which the parasite resides) develops, and nutrient acquisition and ion exchange between the parasite and the erythrocyte cytoplasm is facilitated by the semi-permeable vacuolar membrane (Ansorge et al., 1996; Joiner, 1991).

To reach its destination, the exportome needs to cross two membranes, namely the parasite plasma membrane and the parasitophorous vacuole membrane (PVM). Some of the exported proteins have been found in association with organelles of the erythrocyte cytoskeleton or are deployed to the membrane of the infected erythrocyte, whilst others play a role in the trafficking of proteins to the host cell surface (Haldar et al., 2002, Aravind et al., 2003). Passage through the parasite plasma membrane is achieved through a secretory pathway via the endoplasmic reticulum (ER). For successful trafficking across the PVM, most proteins require a highly conserved *Plasmodium* export element (PEXEL) (Marti et al., 2004) or a vacuolar transport signal (VTS) (Miller et al., 2002; Hiller et al., 2004).

The parasite causes extensive modifications to the host cell. Expressed during the trophozoite stage of the parasite development are flat lamellar structures known as Maurer's Clefts (MCs) and macromolecular complexes known as knobs. Knobs are dense surface protrusions which contain the knob-associated histidine-rich protein (KAHRP), without which knobs would not be present. Vascular endothelium adherence by infected erythrocytes and their rigidity is thought to result from the knob structures. Adherence in the vascular system ensures that infected erythrocytes avoid reaching the spleen where they could be recognised and destroyed by resident macrophages. MCs, in which many exported proteins, including *P. falciparum* exported membrane protein-1 (PfEMP1) have been found, lie beneath the surface of the erythrocyte. They have been implicated in aiding the trafficking of proteins to the surface of the erythrocyte. PfEMP1 renders the infected erythrocyte the ability to adhere to endothelial cells of host capillaries.

1.5.4 The *P. falciparum* exportome

A variety of proteins are exported by the parasite into the host cell cytosol, some of which are trafficked to the erythrocyte membrane. To the parasite, the exportome is useful for enabling cytoadherence which in turn allows the cells to escape immune destruction. Approximately 8% of *P. falciparum* genes encode for proteins containing the PEXEL sequence (Hiller et al., 2004; Marti et al., 2004; Sargeant et al., 2006) and this motif is functionally conserved across all *Plasmodium* species (Marti et al., 2004). In comparison to other *Plasmodium* spp., the *P. falciparum* exportome is at least five times larger.

Transport vesicles fuse with the parasite PM to deliver proteins into the PV (Bannister et al., 2004). The heat-stable antigen (S-antigen) and the serine-rich antigen (SERA) family are some proteins that will remain in the PV, however, other proteins such as KAHRP, the mature parasite-infected erythrocyte surface antigen (MESA) are directed outwards across the PVM

(reviewed by Cooke et al., 2004). It has been suggested that PV-resident proteins as well as those proteins whose destination is beyond the PV, are required to be released into the PV en route (Ansorge et al., 1996; Wickham et al., 2001). However, another suggestion is that proteins destined for the host cell cytosol may be channelled into a specialized secretory compartment within the parasite (Wiser et al., 1997; Cortes et al., 2003). Previous studies report the release vesicles into the PV (Olliaro and Castelli 1997).

On the other side of the PV, interactions between MCs and many exported proteins occur. The MCs might serve as a sorting point from which *P. falciparum* proteins are directed underneath or into the membrane of the infected erythrocyte (Wickham et al., 2001). The Skeleton Binding Protein (SBP1), resident in the MCs, has been implicated in the requirement for the transport of PfEMP1 to the membrane of infected erythrocyte (Cooke et al., 2006; Maier et al., 2007). Hydrophobic N-terminal signal sequences (about 15 amino acids from the N-terminus) characterize proteins destined for sites in the ER, parasite PM, PV and PVM (Lingelbach, 1993), however, several proteins whose destination is beyond the PVM exhibit a longer sequence (up to 30 amino acids) from the N-terminus (Lingelbach, 1993). Proteins such as KAHRP, MESA and Histidine-Rich Protein 2 (HRP2) could make their PV transit as unfolded polypeptides, and later refold to form complexes. The protein complexes would diffuse across the erythrocyte compartment, interact with host erythrocyte proteins and eventually assemble at their appropriate destinations (reviewed by Cooke et al., 2004). The presumption is, however, that the delivery of proteins such as PfEMP1 is via a vesicle-mediated pathway (Taraschi et al., 2001). PfEMP1 is inserted into the MC membrane with the N-terminal domain buried inside the cleft, whilst the C-terminal domain faces the cytoplasm of the host erythrocyte (Kriek et al., 2003). Some soluble proteins whose destination is the erythrocyte membrane seem to be recruited into the MCs. It is at this site where the proteins are assembled into the cytoadhesion complex, and possibly take part in this assembly process

before PfEMP1 is inserted into the host cell membrane (reviewed by Cooke et al., 2004). An N-terminal secretory signal is absent from the Membrane-Associated Histidine-Rich Protein (MAHRP) as is the case for PfSBP1 and PfEMP1. MAHRP exhibits a phenylalanine-rich transmembrane domain which is thought to be inserted into the MCs with the C-terminal exposed to the host cell cytosol (Kriek et al., 2003).

1.5.5 *P. falciparum* Molecular Chaperones

P. falciparum Hsp70s

Six *P. falciparum* Hsp70 (PfHsp70) isoforms have been identified (Peterson et al., 1988; Sargeant et al., 2006). The PfHsp70s have been identified in the cytosol (Peterson et al., 1988), nucleus, mitochondrion and ER (reviewed by Shonhai et al., 2007).

Cytosolic Hsp70 (PfHsp70-1) has been identified in the proteome of the parasitophorous vacuole (Nyalwide and Lingelbach, 2006) and in the MCs (Vincensini et al., 2005), raising the possibility that this protein is exported into the erythrocyte. However, PfHsp70-1 was not detected in the infected erythrocyte in more recent studies, although exposure to heat shock increased the expression of this protein (Pesce et al., 2008). Interestingly, PV- and PVM-resident proteins were also observed in the MC proteome (Vincensini et al., 2005), which disputes the putative MC localization of proteins detected in this study. Previous studies have located PfHsp70-1 in the cytosolic fraction, in the membrane plus organelle fraction and in the nucleus (Kumar et al., 1991; Pesce et al., 2008). PfHsp70-1 has demonstrated chaperone capabilities of preventing protein misfolding and aggregation (Matambo et al., 2004; Shonhai et al., 2005; Shonhai et al., 2008) and has been found to suppress the thermally induced aggregation of malate dehydrogenase (MDH) (Shonhai et al., 2008). Hsp70 proteins are a group of highly ubiquitous proteins and receive significant attention as possible drug targets. Studies screening for Hsp70 inhibitors using the MDH suppression assay have been

conducted. The inhibition of PfHsp70-1 aggregation suppression activity was observed for a total of five compounds (malonganenone A-C, lapachol and bromo-blapachona) that were used (Cockburn et al., 2011). Furthermore, nine compounds have been found to significantly inhibit parasite growth in addition to affecting the ATPase activity of PfHsp70-1 (Chiang et al., 2009). Similarly high compound concentrations were required for PfHsp70-1 activity inhibition studies (Chiang et al., 2009; Cockburn et al., 2011), although the growth of *P. falciparum* using parasite-infected erythrocyte cultures was inhibited at low concentrations (IC_{50} < 2 μM) (Chiang et al., 2009). 15-Deoxypergulation (DSG), an immunosuppressant with anti-malarial activity, has been implicated in modulating ATPase and aggregation suppression activity of PfHsp70-1 by interacting with the EEVD motif (Brodsky 1999; Ramya et al., 2006). DSG had no effect on PfHsp70-2, an Hsp70 protein which lacks an EEVD motif.

PfHsp70-2 is potentially homologous to the ER-resident human Hsp70, human immunoglobulin-binding protein, (BiP) (Kumar and Zheng, 1992; Kappes et al., 1993) and resides in the ER of the parasite (Kumar et al., 1991). PfHsp70-2 may play a role in the assembly of proteins and protein complexes in the ER, as well as transport out of this compartment. PfHsp70-2 has been implicated in the interaction with PfEMP1 in a network of ER chaperones (Pavithra et al., 2007), suggesting that PfHsp70-2 interacts with PfEMP1 outside of the parasite. The trafficking of PfEMP1 has been shown to occur via the ER (Knuepfer et al., 2005).

PfHsp70-3 is a mitochondrial Hsp70 homolog (Sargeant et al., 2006). The presence of PfHsp70-3 has been shown in the PV (Nyalwidhe and Lingelbach, 2006), and this protein is thought to be involved in the import of proteins in the mitochondria (Bauer et al., 2000). The

presence of these proteins in different cellular compartments enables them to undertake different roles within the cell.

PfHsp70-y and PfHsp70-z are Hsp70-like proteins whose ATPase domains are less conserved than their substrate binding domains. PfHsp70-x is thought to be a cytosolic PfHsp70, and possesses a C-terminal EEVN motif that is different to the EEVD motif in human Hsp70 (Sargeant et al., 2006; Shonhai et al 2007). PfHsp70-z is also predicted to localize to the cytosol (Sargeant et al., 2006; Shonhai et al., 2007), whilst PfHsp70-y could potentially localize to the ER (Sargeant et al., 2006; Shonhai et al., 2007).

P. falciparum Hsp40s

Forty three putative *P. falciparum* Hsp40 proteins (PfHsp40s) have been identified (Botha et al., 2007). There are two type I, nine type II, twenty type III and twelve type IV Hsp40s (Botha et al., 2007). The type I PF14_0359 and PFD0462w reside in the parasite; PF14_0359 is believed to be cytosolic (Nakai and Horton, 1999; Botha et al., 2011) and PFD0462w has been localised to the apicoplast. Experiments have indicated that there is an interaction between PF14_0359 and PfHsp70-1 (Pavithra et al., 2007; Botha et al., 2011), indicating that PF14_0359 may be involved in general co-chaperone processes within the parasite. Watanabe (1997), conducted a study where the expression of the mRNAs of four PfHsp40s [Pfj1 (PFD0462w), Pfj2 (PF11_0099), Pfj3 (PF10_0378) and Pfj4 PFL0565w)] was analysed. Upon heat shock at 43 °C for 2 hours, the mRNA levels of Pfj3 were increased, whilst those of Pfj1 and Pfj4 increased to a lesser degree. The mRNA level of the constitutively expressed Pfj2 (Watanabe et al., 1997) was observed to decrease (Watanabe et al., 1997). Up-regulation of Pfj1 protein in the same manner as mRNA levels implies that this protein has a functional role in the heat shock response and the possibility of an association with PfHsp70-1 in the cytoplasm or nucleus. Pfj1 has been shown to stimulate protein refolding activity of PfHsp70-

1 *in vitro* (Misra and Ramachandran, 2009). Interactions between plasmodial J-domains and *E. coli* Hsp70 (DnaK) have been indicated. Nicoll et al (2007) showed that swapping the bacterial J-domains of a thermosensitive *E. coli* strain (OD259) with that of Pfj1 could reverse the thermosensitivity of these bacteria *in vivo*. Pfj1 has also been implicated in the replication of *P. falciparum* apicoplast DNA (Kumar et al., 2010). The upregulation of Pfj3 under heat shock conditions was confirmed (Oakley et al., 2007), as well as that of Pfj4 (Pesce et al., 2008), implying cytoprotective roles of these proteins on the parasite. Pfj4 has been identified in the nucleus and the cytoplasm of parasites at their trophozoite and schizont-stage, where immunoprecipitation studies revealed that anti-Pfj4 antibodies co-localize with PfHsp70-1 (Pesce et al., 2008), suggesting that Pfj4 and PfHsp70 may interact with the parasite. The majority of type III PfHsp40s are believed to reside in the parasite, and are thought to fulfil more specific roles. While two type IV PfHsp40s (PFB0925w and PF11_0443) lack the PEXEL sequence and are predicted to reside in the parasite. The PEXEL motif has been identified in the Pfj3 protein, suggesting that it may be exported to the cytoplasm of the infected host cell (Sargeant et al., 2006).

The PEXEL motif

The PEXEL motif (Marti et al., 2004) or the VTS (Hiller et al., 2004) is encoded by the RxLxQ/E consensus and is a common feature between parasite-encoded proteins that are trafficked to the erythrocyte. The PEXEL motif was discovered when N-terminal sequences of proteins known to be exported from the PV to the erythrocyte were aligned, (Marti et al., 2004; Hiller et al., 2004). Studies reveal that the PEXEL is necessary for export of proteins beyond the VT into the erythrocyte (Marti et al., 2004).

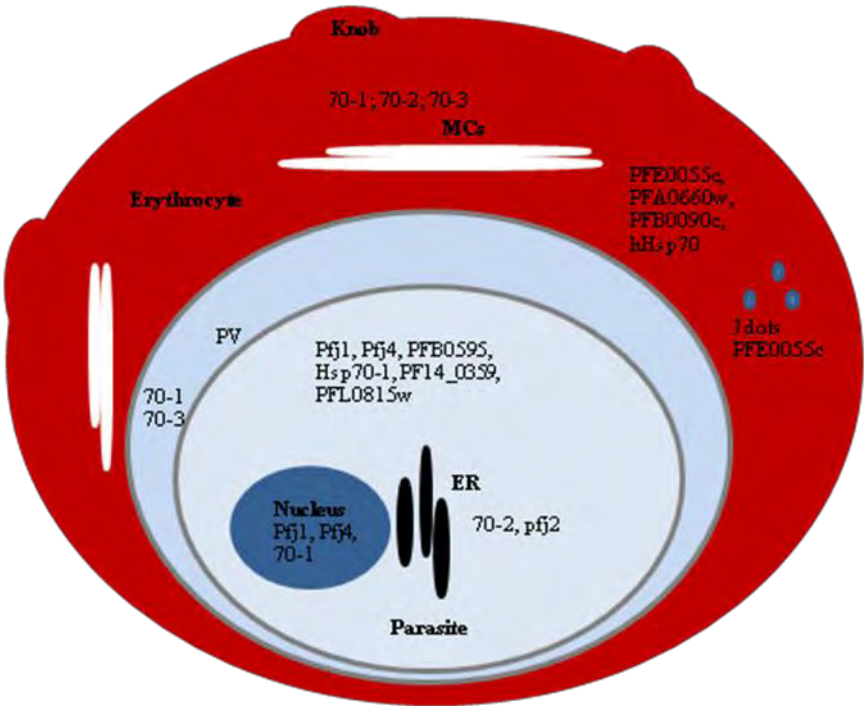

Figure 1.6: Schematic representation of chaperones in the *P. falciparum*-infected erythrocyte.
The Figure shows the *P. falciparum* parasite encapsulated within a parasitophorous vacuole. Organelles, compartments, parasite-resident proteins and those that are exported are described in detail in sections below.

1.5.5.1.1 *Exported Hsp40 proteins*

Exported proteins contain an N-terminal signal sequence (SS) which is responsible for co-translational translocation into the ER (Haldar et al., 2002; Wicknam et al., 2001). Some PfHsp40 proteins were identified as part of the exportome prior to the discovery of the PEXEL motif. Such proteins include the ring-infected erythrocyte surface antigen protein family (RESA) (Da Silva et al., 1994; Foley et al., 1991). Proteins belonging to this family have been identified as exported to the host cell membrane where they interact with components of the membrane skeleton, and have also been functionally implicated in facilitating knob formation (Foley et al., 1991; Da Silva et al., 1994). It should be noted that RESA proteins lack the HPD motif in their J-domain and so have been classified as type IV PfHsp40s. Of the chaperones in the *P. falciparum*, only those belonging to the Hsp40 class contain the PEXEL motif. Nineteen PfHsp40 proteins are known to contain the PEXEL

(Botha et al., 2007), suggesting that they are exported into the erythrocyte. Of the 9 identified type II Hsp40s, PFA0660w, PFB0090c, PFE0055c and PF11_0099 contain the PEXEL motif and PFA0660w, PFB0090c and PFE0055c are predicted to be exported to the erythrocyte cytosol (Botha et al., 2007; Maier et al., 2008).

Table 1.1 *Plasmodium falciparum* type II Hsp40 proteins, prediction of export and localisation

PlasmoDB ID	Accession number	Protein description	PEXEL	Localisation
PFA0660w	NP_703333.1	DnaJ homolog, Sis1 family	Yes	Erythrocyte
PFB0090c	NP_472947.2	Hypothetical protein	Yes	Erythrocyte
PFE0055c	NP_703357.1	Heat shock protein, putative	Yes	Erythrocyte
PF11_0099	NP_7000963.1	DnaJ homolog, Pfj2	Yes	ER
MAL13P1.277	NP_705450.1	DnaJ-like protein, putative	No	Mitochondrion
PF14_0137	NP_702025.1	Hypothetical protein	No	Apicoplast
PFB0595w	NP_473047.1	Sis1 homolog	No	Not determined
PFF1415c	XP_966274.1	DnaJ protein	No	Apicoplast
PFL0565w	NP_701478.1	DnaJ homolog, Pfj4	No	Not determined

Four out of 20 type III Hsp40s bear the PEXEL motif (Botha et al., 2007), and they are thought to perform more specialised roles in *P. falciparum* due to their diversity (Kelley, 1998). Of the 12 that have been identified, 11 type IV Hsp40s are classified as having the PEXEL motif (Botha et al., 2007) and are predicted to be exported. Gene knock-out studies have been conducted where loss-of-function mutants encoding proteins predicted to be exported were constructed. PF11_0034 and PF11_0509 could not be disrupted, suggesting that they play an important role in parasite survival (Maier et al., 2008). No PfHsp40 protein was shown to be required for the surface expression of PfEMP1 (Maier et al., 2008), although RESA, PFA0110w and PFB0920w have been shown to affect membrane rigidity of infected erythrocytes (Maier et al., 2008). Whilst it was possible to disrupt the genes encoding PFE0055c and PFB0090c, it was not possible to obtain PFA0660w loss of function parasites. PFE0055c has been identified in the MCs (Bhattacharjee et al., 2008) as well as in the host

cell cytoplasm (Hiller et al., 2004). Kulzer et al (2010) conducted a study which showed that PFE0055c does not co-localise with SBP-1, a known MC-resident protein.

1.5.5.1.2 *PFE0055c protein*

PFE0055c is a putative malaria Hsp40 protein of 413 amino acids. It contains a PEXEL motif and is one of the four type II Hsp40s that are predicted to be part of the *P. falciparum* exportome (Sargeant et al., 2006; Bhattacharjee et al., 2008). PFE0055c exhibits a high degree of sequence identity with two other exported type II Hsp40s (PFA0660w and PFB0090c). Evidence suggesting the presence of PFE0055c in the parasitophorous vacuole, cytoplasm and MCs has been provided (Nyalwidhe and Lingelbach, 2006; Vincensini et al., 2006; Bhattacharjee et al., 2008). Human homologues have been identified for several PEXEL-containing type II Hsp40s including PFE0055c. The export of these proteins to the erythrocyte implies that they may functionally replace DnaJB4 in humans (Botha et al., 2007). In a recent study conducted by Kulzer et al., (2010), PFE0055c was found to associate with structures found in the erythrocyte cytosol termed "J-dots", which might be involved in trafficking parasite-encoded proteins through the erythrocyte cytosol. PFE0055c, along with 87 other proteins, was identified from peripheral blood of patients infected with *P. falciparum* (Acharya et al., 2009), implying a functional role in the host cell. In gene knockout studies conducted by Maier et al., (2008), it was found that the PFE0055c gene may not be essential for parasite survival *in vivo*. While PFE0055c may not be essential, it may be important for pathogenesis as it may possibly function with human Hsp70 in assisting the transport of other plasmodial proteins. The study of PFE0055c is part of a larger project that includes analyses of PFA0660w and PFB0090c by other members of the group.

1.6 Problem statement

Plasmodium species export Hsp40 proteins into the infected erythrocyte, but not Hsp70 proteins. If Hsp40 is acting as a co-chaperone to Hsp70, it must interact with human Hsp70 within the infected erythrocyte. Studying this interaction can provide fundamental insight into Hsp40-Hsp70 interaction, as well as possibly being a target for drug design.

1.7 Hypothesis

The type II exported PfHsp40 PFE0055c protein interacts with human Hsp70 proteins in the infected erythrocyte cytosol, thereby facilitating the transport, folding, and assembly of other exported malaria proteins, some of them being virulent factors. Identifying the interaction of PFE0055c with Hsp70 may help to define its functions.

1.8 Research objectives

1.8.1 Broad objective

The broad objectives of this project are the biochemical and cell biological characterization of PFE0055c, its interaction with human Hsp70, and the role of those interactions in the folding and assembly of other malaria proteins potentially important for the survival and establishment of the parasite in infected erythrocytes.

1.8.2 Specific objectives

- To conduct a preliminary bioinformatic analysis of PFE0055c;
- To over-express and affinity purify recombinant PFE0055c and human Hsp70 using a heterologous expression system;
- To analyse the ability of PFE0055c to stimulate the ATPase activity of human Hsp70 (hHsp70A1A).

Chapter 2 : Methods and Materials

2.1 Materials

Yeast, tryptone, sodium chloride, magnesium chloride and calcium chloride were purchased from Merck (Germany). *E. coli* (M15[pREP4]) was obtained from Qiagen (USA). *E. coli* (BL21) was obtained from Stratagen (USA). Ampicilin, kanamycin and isopropyl-1-thio-β-D-galactopyranoside (IPTG) were purchased from Roche (Germany). The pQE30 plasmid was purchased from Qiagen (USA). The pQE30-PFE0055c construct was synthesised by GenScript (USA) and obtained from Taryn Bodill (Bodill MSc thesis, 2009). The pMSHSP construct was obtained from Jeffrey Brodsky (University of Pittsburgh, USA). Alfalfa Hsp70 was purchased from Fermentas (USA). Spin Miniprep kit was purchased from Qiagen (USA). Tris and EDTA were purchased from Sigma (USA). *Bam*H1 and *Pst*1 were obtained from New England Biolabs, UK. Bromophenol blue, glycerol and agarose were purchased from Merck (Germany). Lambda DNA was purchased from Promega (USA). Polyclonal goat anti-PFE0055c antibodies were produced by GenScript (USA) and obtained from Taryn Bodill (Bodill MSc thesis, 2009). Donkey anti-goat IgG horseradish peroxide (HRP)-linked secondary antibodies were purchased from Promega (USA). Mouse anti-*His* primary antibody and anti-mouse IgG HRP-linked species-specific whole secondary antibody were purchased from GE Healthcare (UK). Coomassie G-250, potassium chloride (KCl), magnesium chloride ($MgCl_2$), disodium hydrogen phosphate (Na_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), 2-mercaptoethanol, hepes and Tween-20 were purchased from Merck (Germany). Phenylmethylsulfonyl fluoride (PMSF), sodium dodecyl sulphate-polyacrylamide (SDS), imidazole, Ponceau S, urea and N,N,N',N'-tetramethylethylenediamine (TEMED) were purchased from Sigma (USA). Ammonium persulphate (APS) and lysozyme were purchased from Roche (Germany). Nitrocellulose membrane was obtained from BioRad, (USA). The ECL advance western blotting detection Kit was purchased from BioRad (USA). Fast flow chelating sepherose was purchased from GE Healthcare (UK). Nickel (II) sulphate hexahydrate was purchased from Fluka (Germany). Hydrochloric acid, ascorbic acid, sodium

citrate and ammonium molybdate were purchased from Merck (Germany). Dithiothreitol (DTT) was purchased from Fermentas (USA). ATP was purchased from Sigma (USA).

2.2 Methods

2.2.1 Bioinformatics Analysis

P. falciparum protein sequences were obtained from PlasmoDB (www.PlasmoDB.org) (Bahl et al., 2003). PlasmoDB is a genomic database for the genus Plasmodium. Other protein sequences were downloaded from National Centre for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov) (Shi et al., 2001). Multiple sequence alignments were generated using ClustalW (Thompson et al., 1994). ClustalW is a sequence alignment program for DNA and proteins. The 3-D models were visualized using PyMOL (DeLano, 2002). PyMOL is a graphics tool that enables the 3-D visualization of proteins and small molecules.

2.2.2 Preparation of Competent *E. coli* Cells

A single colony of *E. coli* (M15[pREP4]) cells was used to incubate 5 ml of yeast tryptone YT broth (1 % (w/v) yeast, 1.6 % (w/v) tryptone and (0.5 % (w/v) sodium chloride) and grown at 37 °C overnight with shaking. The culture was diluted 1/200 in fresh YT broth and allowed to grow to early log phase (A_{600} between 0.3 – 0.6). The culture was collected by centrifugation at 5000x*g* at 4 °C for 5 minutes. The cell pellet was placed on ice and resuspended in 50 ml of ice-cold 0.1 M $MgCl_2$ for 20 minutes. The cells were centrifuged at 5000x*g* for 5 minutes at 4 °C and the pellet resuspended in 25 ml of ice-cold 0.1 M $CaCl_2$ for 2 hours. After another centrifugation at 5000x*g* at 4 °C for 5 minutes, the cells were resuspended in 5 ml of ice-cold 0.1 M, $CaCl_2$ including 30 % (v/v) glycerol. The cells were stored in aliquots at -80 °C. The preparation of competent *E. coli* (BL21) cells and their storage was performed according to the protocol described above.

2.2.3 Transformation of Competent *E. coli* (M15[pREP4]) Cells and *E. coli* (BL21) Cells

The heat shock method (Chung et al., 1989) was used to transform *E. coli* (M15[pREP4]) cells with pQE30-PFE0055c plasmid DNA, whilst *E. coli* BL21 was transformed with pMSHSP plasmid DNA . The *E. coli* cells were incubated on ice with the plasmid DNA (100 ng) for 30 minutes. The cells were heat-shocked for 60 seconds at 42 °C, incubated in ice for 2 minutes and 900 µl of YT broth added and the mixture incubated for 1 hour with shaking. An aliquot (100 µl) of the cells was plated onto YT agar plates (YT broth with 1.5 % (w/v) agar containing 100 µg/ml of ampicillin for the BL21 cell line; and 100 µg/ml of ampicillin and 50 µg/ml of kanamycin for the [M15pREP4] plasmid) and incubated overnight at 37 °C.

2.2.4 Plasmid DNA Extraction

A single *E. coli* BL21 transformant was used to inoculate 5 ml of YT broth containing 100µg/ml ampicillin and grown overnight at 37 °C with shaking. Plasmid DNA was isolated using the QIAprep spin miniprep kit according to the manufacturer's instructions. TE buffer (50 µl; 10 mM Tris, pH 8: 1 mM EDTA) was used to elute the plasmid DNA.

2.2.5 Restriction Digestion of Plasmid DNA

Restriction endonucleases *Bam*H1 and *Pst*1 were used to digest the pQE30-PFE0055c plasmid DNA (10 – 100 ng) in a total volume of 20 µl. One reaction remained enzyme free to serve as the uncut sample. The samples were incubated at 37 °C for 2 hours. Gel loading buffer (0.25 % (w/v) bromophenol blue in 30 % (v/v) glycerol) was used to stop the reactions and the samples were analysed on a 0.8 % (w/v) agarose gel in TAE buffer [45 mM Tris pH 8, 45 mM acetic acid, 1 mM ethylenediaminetetraacetic acid (EDTA)].

2.2.6 Agarose Gel Electrophoresis

Agarose gels [0.8 % (w/v)] containing 0.5 µg/ml ethidium bromide were prepared using TAE buffer. Samples were resolved for 1 hour at 100 V. DNA bands were visualized using ultra violet (UV) radiation and imaged using a ChemiDoc (BioRad, USA).

2.2.7 Protein Expression of *His_6*-PFE0055c in *E. coli* (M15[pREP4]) Cells

A single *E. coli* (M15[pREP4]) colony transformed with the pQ30-PFE0055c construct was used to inoculate YT broth (25 ml containing 100 µg/ml ampicillin and 50 µg/ml kanamycin) and incubated overnight at 37 °C with shaking. The overnight culture was diluted into fresh broth (225 ml) containing 100 µg/ml ampicillin and 50 µg/ml kanamycin and grown to a density of A_{600} between 0.6 – 0.8. To induce protein expression, isopropyl-1-thio-β-D-galactopyranoside (IPTG) (1 mM) was added to the culture. Aliquots were taken before induction and every hour after induction for a period of 5 hours plus overnight. Each aliquot was centrifuged for 60 seconds at 16000x*g*. The pellets were resuspended in phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM, Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4), in a volume equivalent to A_{600}/0.5*150 µl and treated with SDS-PAGE loading buffer (0.063 M Tris, 10 % (v/v) glycerol, 2 % (w/v) SDS, 2.5 % (w/v) bromophenol blue, 5 % (v/v) 2-mercaptoethanol). The samples were boiled for 5 minutes and analysed by SDS-PAGE and western analysis.

2.2.8 Protein Expression of hHsp70A1A-*His_6* in *E. coli* (BL21) cells

A single *E. coli* (BL21) colony transformed with the pMSHSP construct, that encodes the human Hsp70A1A protein with a C-terminal hexahistidine tag, was used to inoculate YT

broth (25 ml containing 100 μg/ml ampicillin) and was incubated overnight at 37 °C. The overnight culture was diluted into fresh broth (225 ml) containing 100 μg/ml ampicillin and grown to a density of A_{600} between 0.6 – 0.8. To induce protein expression, isopropyl-1-thio-β-D-galactopyranoside (IPTG) (1 mM) was added to the culture. Aliquots were taken before induction and every hour after induction for a period of 5 hours. Each aliquot was centrifuged for 60 seconds at 16000x*g*. The pellets were resuspended in PBS and treated with 5x SDS-PAGE loading buffer as described previously. The samples were boiled for 5 minutes and analysed by SDS-PAGE and western analysis.

2.2.9 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were analysed using a discontinuous gel composed of 4 % stacking (0.125 M Tris-HCl, pH 6.8, 0.1 % (w/v) SDS, 4 % (v/v) acrylamide/bis-acrylamide, 0.2 % (w/v) ammonium persulphate [APS], 0.004 % (v/v) N,N,N',N'-tetramethylethylenediamine [TEMED]); and 12 % resolving gel (0.375 Mtris-HCl, pH 8.8, 0.1 % (w/v) SDS, 12 % (v/v) acrylamide/bis-acrylamide, 0.2 % (w/v) APS, 0.004 % (v/v) TEMED) at 170 V for approximately 1 hour in SDS-PAGE running buffer (25mM Tris, 192 mM glycine, 0.1 % (w/v) SDS, pH 8.3). Coomassie staining solution (0.2 % (w/v) Coomassie G-250, 40 % (v/v) methanol, 7 % (v/v) glacial acetic acid) was used to stain protein bands for visualization and a destain solution (40 % (v/v) methanol, 7 % (v/v) glacial acetic acid) was used to destain the gel.

2.2.10 Western Analysis

Proteins were transferred from the SDS gel onto a nitrocellulose membrane (BioRad, USA). The transfer was allowed to proceed for 1 hour at 100 V on ice. The membrane was stained with Ponceau S stain (0.1 % (w/v) Ponceau S in 1 % (v/v) glacial acetic acid) to confirm protein transfer and destained with distilled water and subsequently blocked overnight at 4 °C

with 5 % (w/v) fat free milk powder in TBS-T (150 mM NaCl, 50 mM Tris, pH 7.6, 0.1 % (v/v) Tween-20). The membrane was incubated, with a primary antibody (as indicated in figure legends) in blocking solution, at room temperature on a rocker for 1 hour, followed by four washes with TBS-T at 15 minute intervals. This was followed by secondary antibody incubation (as indicated in figure legends) and subsequent washing under the same conditions as the primary antibody. The ECL advance western blotting detection Kit and a BioRad ChemiDoc system (BioRad, USA) were used for protein detection and image acquisition respectively.

2.2.11 Preparation of Ni^{2+} Chelating sepharose beads

Sepharose beads were charged with Ni^{2+} according to the manufacturer's instructions. The beads were resuspended in 1ml lysis buffer: (10 mM Tris pH 7.5, 300 mM NaCl, 50 mM Imidazole, 1 mM phenylmethylsulfonyl fluoride [PMSF]) for native purification and (8M urea, 300 mM NaCl, 100 mM Tris pH 8, 10 mM imidazole, 1 mM PMSF) for purification under denaturing conditions.

2.2.12 Purification of *His_6*-PFE0055c under Denaturing Conditions

E. coli (M15[pREP4]) competent cells were transformed with the pQE30-PFE0055c plasmid. A fresh colony was picked for protein induction and protein induction performed as described previously. The cells were harvested at 4 hours post induction with IPTG (1 mM). The cells were centrifuged at 6000x*g* for 30 minutes at 4 °C. The pellets were suspended in denaturing lysis buffer (8 M urea, 300 mM NaCl, 100 mM Tris pH 8, 10 mM imidazole, 1 mM PMSF) and stored overnight at -20 °C. Lysozyme (1 mg/ml) was added to thawed cells on ice and incubated for 1 hour at 37 °C. The cells were sonicated for 5 x 30 seconds at 50 % amplitude using the Vibra Cell (Sonics Materials, USA) and centrifuged at 16000x*g* for 30 minutes at 4

°C. An aliquot (80 µl) of the supernatant cleared lysate was prepared for SDS-PAGE and western analysis. The remaining supernatant was transferred to nickel-charged sepharose beads and incubated overnight on ice with shaking. The sample was centrifuged at 1300x*g* for 2 minutes and 80 µl of the supernatant (flow through) was reserved for SDS-PAGE and western analysis. The resin was resuspended in 3 ml of wash buffer (containing varying concentrations of urea (6 mM, 4 mM and 2 mM in 300 mM NaCl, 100 mM Tris, 10 mM imidazole, 1 mM PMSF). After each wash, the resin was collected by centrifugation for 2 minutes at 1300x*g* and 80 µl samples were reserved for SDS-PAGE and western analysis. Purified protein was eluted 3 times with 3 ml elution buffer (300 mM NaCl, 100 mM Tris, 1 M Imidazole, 1 mM PMSF). The concentration of purified protein was determined using the Nanodrop (BioRad, USA). The protein was dialysed overnight in 500 ml dialysis buffer (300 mM NaCl, 100 mM Tris pH 7.5, 1 mM PMSF). Dialysis was continued in fresh dialysis buffer for a further five hours. The protein was stored at 4 °C.

2.2.13 Purification of Human Hsp70A1A under Native Conditions

E. coli BL21 competent cells were transformed with the pMSHSP plasmid. A fresh colony was picked for protein induction and induction performed as described previously. To purify the protein, the cells were harvested by centrifugation at 5000x*g*, for 10 minutes at 4 °C. The supernatant was discarded and the pellet resupsended in 10 ml TEK_{50} buffer (20 mM Tris, pH 8; 0.1 mM EDTA; 50 mM KCl). The cell pellet was obtained by centrifugation at 5000x*g* for 10 minutes at 4 °C. The supernatant was discarded and the weighed pellets were kept at -80 °C overnight. The pellet was thawed and subsequently resuspended in a volume equal to 3 ml TEK_{50} buffer per 1 g of cells. Lysozyme (1mg/ml) and 1 mM PMSF were added and the pellet was incubated on ice for 30 minutes. Three freeze-thaw cycles were carried out using liquid nitrogen and a 30 °C water bath. The cells were sonicated 7 times for 15 minutes at 50

% amplitude using Vibra Cell (Sonics Materials, USA) each, with incubating on ice for 30 seconds in between. Lysed cells were centrifuged at 13000x*g* at 4 °C for 30 minutes and the supernatant collected. All the pellets were resuspended in a total of 10 ml TEK_{50}, pooled and sonicated 5 times for 15 seconds at 50 % amplitude using Vibra Cell (Sonics Materials, USA) each, with 30 second incubation on ice in between sonications. The lysed cells were centrifuged at 13000x*g* for 30 minutes. The supernatants from the two sonications were pooled and loaded onto nickel-charged sepharose beads and allowed to bind overnight on ice with shaking. An aliquot of the supernatant (80 µl) was reserved for SDS-PAGE and western analysis (cleared lysate). To remove the unbound fraction, the beads were collected by centrifugation at 4000x*g* for 60 seconds at 4 °C. A sample (80 µl) of the supernatant (flow through) was taken for SDS-PAGE and western analysis. The beads were washed three times with wash buffer (300 mM NaCl, 10 mM Tris pH 7.5, 50 mM imidazole, 1 mM PMSF) and centrifuged for 2 minutes at 4000x*g* at 4 °C. Human Hsp70 A1A was eluted 3 times using elution buffer (3 ml; 300 mM NaCl, 10 mM Tris pH 7.5, 1 M imidazole, 1 mM PMSF). The concentration of purified protein was detected using the Nanodrop (BioRad, USA). The protein was dialysed overnight in 500 ml dialysis buffer (300 mM NaCl, 10 mM Tris pH 7.5, 1 mM PMSF). Dialysis was continued in fresh dialysis buffer for a further five hours. The protein was stored at 4 °C.

2.2.14 ATPase Assay

ATPase assay reactions were performed in microcentrifuge tubes using ATPase buffered Solution (10 mM Hepes pH 7.4, 10 mM $MgCl_2$, 20 mM KCl, 0.5 mM DTT). Different concentrations and combinations of chaperones and co-chaperones (0.4 µM and 1 µM of PFE0055c; 0.4 µM and 1 µM of hHsp70A1A; 0.05 µM alfalfa Hsp70) were tested (as indicated in figure legends). The reactions were equilibrated to 37 °C for 5 minutes previous the addition of 0.6 mM ATP. At varying time points (0, 15 minutes, 30 minutes, 60 minutes,

120 minutes, 180 minutes), 3 aliquots (50 µl) of the reaction were taken and added to 50 µl of 10 % (w/v) SDS in order to stop the reaction. Phosphate standards (KH_2PO_4) were prepared at concentrations ranging from 0.2 nmol/µl to 8 nmol/µl. For the development of the colorimetric reaction, 50 µl of 1 % (w/v) ammonium molybdate in 1 N HCl, 50 µl of 6 % (w/v) ascorbic acid, 125 µl of 2 % (w/v) sodium citrate and 2 % (v/v) acetic acid were added to the samples. The microtitre plate was incubated at 37 °C for 3 hours and absorbance read at 850 nm. The absorbance readings of ATPase activity were converted to nmol of inorganic phosphate (P_i) using the phosphate standard curve and subsequently finalized to specific activity, taking into consideration the concentration of the protein in mg/ml and the time of incubation of the ATPase reaction. Controls included reactions that contained the chaperones and co-chaperones but lack ATP, as well as reactions in which ATP was incubated in the absence of chaperones and/or co-chaperone. The ATPase activity calculated from the control that did not contain proteins was regarded as spontaneous breakdown of ATP to ADP and Pi, as opposed to enzyme catalysed hydrolysis of ATP. This value was subtracted from the final ATP hydrolysis activities of the reactions containing proteins. Microsoft Excel was used for data analysis.

Chapter 3 : Results

3.1 Bioinformatic Analysis of PFE0055c

The *P. falciparum* genome has been sequenced and revealed a diverse range of molecular chaperones. It encodes at least forty three members of the Hsp40 family and six members of the Hsp70 family (Botha et al., 2007). The *P. falciparum* Hsp70 (PfHsp70) proteins are homologous to cytosolic, mitochondrial and endoplasmic reticulum (ER) Hsp70s. Interestingly, none of the Hsp70 proteins contain a PEXEL/HT (Plasmodium export element/host targeting signal) sequence motif, suggesting that they are all parasite-resident. On the contrary, nearly half of the Hsp40 proteins have been classified as being PEXEL/HT motif positive; giving rise to the possibility that they are exported, thereby play a role in infected-erythrocyte remodelling. The field of bioinformatics is an application of computational studies to molecular biology with the aim of gaining insight in protein structure and function through *in silico* analyses of nucleic acid sequences. Bioinformatics is a useful tool for the interpretation of biological data (Luscombe et al., 2001) and can be used to predict possible functions of novel proteins. A bioinformatic analysis of PFE0055c, a type II Hsp40, was investigated.

3.1.1 Alignment of PFE0055c amino acid sequence with canonical Hsp40

In this study, the amino sequence of PFE0055c was aligned with that of DnaJ, an *E. coli* type I Hsp40; as well as with that of Sis1, a *S. cerevisiae* type II Hsp40. The amino acid sequences of the nine type II PfHsp40s were also analysed. Hsp40 proteins function as co-chaperones for Hsp70 and can be classified into four different types according to their structural features. All Hsp40 proteins exhibit a signature J-domain, essential for interaction with partner Hsp70 proteins. The stimulation of the ATPase activity of Hsp70 by Hsp40 is dependent on the HPD tripeptide motif, which is found in between the second and third helices of the J domain. Following the J-domain is a G/F-rich region and a C-terminus after the cysteine-repeat zinc

finger region. All four regions are present in type I Hsp40proteins, whilst type II Hsp40s lack only the zinc finger region. Type III Hsp40s comprise of the C-terminus in addition to the J-domain (Cheetham and Caplan, 1998). Type IV Hsp40s are a newly described subtype of Hsp40 in which the HPD motif is corrupted (Botha et al., 2007).

Comparison of the PFE0055c sequence with DnaJ, a type I Hsp40 protein from Escherichia coli

The amino acid sequence of a typical type I Hsp40 (*E. coli* DnaJ) was aligned with that of PFE0055c. Classification of proteins as type I to type III Hsp40s depends on the degree of conservation with *E. coli* DnaJ (Cheetham and Caplan, 1998). The sequence alignment of PFE0055c and *E. coli* DnaJ, a type I Hsp40, is shown in Figure 3.1.

The alignment identified that PFE0055c contains an N-terminal extension which was absent from DnaJ. Both DnaJ and PFE0055c included the J-domain (highlighted in green in Figure 3.1), located towards the N-terminus of the protein. The highly conserved HPD motif, known to be crucial for interaction with partner Hsp70 proteins, was conserved in the J-domains of DnaJ and PFE0055c (Figure 3.1; highlighted in red). The J-domains between DnaJ and PFE0055c share a 55 % sequence identity. A G/F-rich region is observed in DnaJ (Figure 3.1; highlighted in blue) and contains the highly conserved DIF motif (Figure 3.1; shaded in black) which is lacking in the G/F-rich region of PFE0055c (Figure 3.1; highlighted in blue). Cysteine-repeats (Figure 3.1; highlighted in orange) are observed in DnaJ but are lacking in PFE0055c. The C-terminus of PFE0055c exhibited 62 % identity with that of DnaJ.

Figure 3.1: Multiple Sequence alignment of the amino acid sequence of PFE0055c with that of *E. coli* DnaJ.
The alignment was generated using ClustalW. DnaJ is the *E. coli* type I Hsp40 protein and represents a canonical type I Hsp40. J domains are highlighted green, G/F-rich region is highlighted blue; and cystein-rich repeats are highlighted orange. The HPD motif within the J domain is highlighted red. The KFK motif, located on helix III of the J domain, is shaded black. The DIF motif, which PFE0055c lacks, is also shaded black. The stars indicate identical residues, the double dots indicate a high degree of similarity and single dots indicate a lesser degree of residue similarity residues.

Comparison of the PFE0055c sequence with Sis1 from Saccharomyces cerevisiae

The amino acid sequence of a typical type II Hsp40 (Sis1) was aligned against that of PFE0055c and the results are shown in Figure 3.2.

Figure 3.2: Sequence alignment of the amino acid sequence of PFE0055c with Sis1.

The alignment was generated using ClustalW. Sis1 is a type II Hsp40 from *S. cerevisiae*. J domains are highlighted green, G/F rich regions highlighted blue. The HPD motif is highlighted red within the J domain. The KFK motif is shaded black. The stars indicate identical residues, the double dots indicate similar residues.

Sis1 and PFE0055c both comprise of the J-domain (Figure3.2), highlighted in green and similar to DnaJ, the N-terminal region is extended in PFE0055c compared with Sis1. The highly conserved HPD motif is observed in the J-domains of both these proteins. The J-domains between these two type II Hsp40s share a sequence identity of 44 % (Figure 3.2). A G/F-rich region was observed in both PFE0055c and Sis1 (highlighted in blue), which contains the highly conserved DIF motif which the G/F-rich region of PFE0055c lacks (Figures 3.2). The C-terminus of PFE0055c exhibited a greater degree of sequence similarities when compared to Sis as opposed to DnaJ.

3.1.2 Comparison of the PFE0055c sequence with other type II Hsp40s from *Plasmodium falciparum*.

The amino acid sequence of PFE0055c was aligned with that of eight PfHsp40 proteins that have been classified as being type II Hsp40s. The alignment is shown in figure 3.3.

```
PFE0055c            GCRKKLKVTRKRFVGL--------NSYEDNT-FITV-DVKP------GWSEGTKINFHGE
PFB0595w            GCKKKLKITRKRFMGT--------KSYEDDN-YVTI-DVKA------GWKDGTKITFYGE
PF11_0099           -----L-KLRKKFDGYITFA---VVNCQEENMLCRKYNVKSLPQLILMRSDKTYETFYGN
PF14_0137           EEDTEV-YVSTFCEYVFENNSKSIKNYDDAVLFCAR-LVDG------KMNNASNFSWREE
MAL13P1.277         QY----------------------------------ILKS------HFKEKPILIYDAY
PFL0565w            GGSCFTSVETSTSNG--------------GK-FKNR-VVKT------STSKSTSI-----
                                                      :.          .

PFF1415c            GKQ-----EIGY-ENGDIIFIV---QTKKHKIYER-------------VNNDLHQIYEIS
PFA0660w            GDQ-----SSPDSYPGDLVLVL---QTKKHSKFVR-------------KSRDLYYRHIIT
PFB0090c            GDQ-----TSPMSNPGDLVFTI---KTVDHDRFVR-------------SYNDLIYRCPIT
PFE0055c            GEQ-----SSPNEQPGDLVFII---KTKPHDRFIR-------------EGNNLIYKCYLP
PFB0595w            GDQ-----LSPMAQPGDLVFKV---KTKTHDRFLR-------------DANHLIYKCPVP
PF11_0099           RTDENLTYFIKNNIPSAIIE---CNNQKKLDNFLTQNIEIPKVLFFISHNDNIVMLKALS
PF14_0137           KSSLD—ITNMYELDDDKVFDK---QYDKDDIFYS-------------------------
MAL13P1.277         GRA-----F-----------LV-------------------------------------
PFL0565w            -------------INGKRVTRIETVKTLPNGTVER-------------------------

PFF1415c            LK--------------------------------------DA-------LIGFSKNLEHIS
PFA0660w            LE--------------------------------------QS-------LTGFDFVIKSLD
PFB0090c            LE--------------------------------------QA-------LTGHKFTIITLD
PFE0055c            LD--------------------------------------KA-------LTGFQFSIKSLD
PFB0595w            LD--------------------------------------KA-------LTGFQFIVKSLD
PF11_0099           LEFKKRINIGIIYNTNYSVMKLFKKKNIKTPSLLLVDDIDSLSGDLTQLKNFDFNILSLK
PF14_0137           ---------------------------------------------------LLYNIIENN
MAL13P1.277         ----------------------------------------D-------IHGRKFRASEFD
PFL0565w            ----------------------------------------T-------VTER----EEDD
                                                                          .

PFF1415c            GKPININK—Q-NVTFHNE------VLR—VQNKGMPIKNS-NKFGDLYIKF—LIQFPK
PFA0660w            NRDIHIQI—D-EVVKPDT------KKV—IKNEGMPYSRDPSIRGNLIVEF—DIIYPN
PFB0090c            NRDIDIQV—D-EIVTPLT------TRV—ITSEGMPYMENPKMKGNLIIEF—DIIFPK
PFE0055c            NRDINVRV—D-DIINPNS------KKI—ITNEGMPYSKSPSVKGDLFIEF—DIVFPK
PFB0595w            NRDINVRV—D-DIVTPKS------RKI—VAKEGMPSSKYPSMKGDLIVEF—DIVFPK
PF11_0099           LSHIVAQNRLKNNLYGHVT---SYQE----LTK—KKYESGQCHEKDSQICFFILKLLKK
PF14_0137           KNQIDLKIPKK-ELCRRFDWSRWYTTAILEKNTEEKNFVESNATKGIIFSSVLYFIGLVS
MAL13P1.277         KY----------------------------------------------------------
PFL0565w            RGNININRQ---------------------------LPAHELRRNKR--------------

PFF1415c            Q-------LTDEQKKVLADLL---------------------------------------
PFA0660w            T-------IKKEQKKLIKEIFKESY-----------------------------------
PFB0090c            K-------LSDEQKELIKEALGGNGF----------------------------------
PFE0055c            K-------LSPEQKRTLKETLENTY-----------------------------------
PFB0595w            S-------LTSEKKKIIRETLANTF----------------------------------
PF11_0099           NYKSFDEDIKKVANKFSSDPLKILYINIYQQPYILDSFGLSNNIQYSNGLILVAFRPKRQ
PF14_0137           H-------LVSK------------------------------------------------
MAL13P1.277         ----------------------------------------------------------
PFL0565w            ----------------------------------------------------------

PFF1415c            -------------------------------------------------
PFA0660w            -------------------------------------------------
PFB0090c            -------------------------------------------------
PFE0055c            -------------------------------------------------
PFB0595w            -------------------------------------------------
PF11_0099           KFKVYDGDVNVENVHKFVDNVVSGGIPINQNIKRSLKFVHVEQYDDEL
PF14_0137           -------------------------------------------------
MAL13P1.277         -------------------------------------------------
PFL0565w            -------------------------------------------------
```

Figure 3.3: Sequence alignment of the amino acid sequences of all type II PfHsp40 proteins.

The alignment was generated using ClustalW. Nine identified Hsp40 proteins were aligned. J domains are highlighted green, G/F rich regions highlighted blue. The HPD motif is highlighted red. The KFK motif is shaded black. The stars indicate identical residues, the double dots indicate a high degree of similarity and single dots indicate a lesser degree of residue similarity.

The amino acid sequence of PFE0055c was aligned with that of eight PfHsp40 proteins that have been classified as being type II Hsp40s (Figure 3.3). PFB0595w, MAL13P1.277 and PFL0565w lack the N-terminal extension while all of the type II PfHsp40 proteins contain the J-domain (highlighted in green) containing the highly conserved HPD motif (highlighted in red). PFA0660w, PFE0055c, PFB0595w and MAL13P1.227 all contain the KFK motif (shaded black) within the J-domain. PF11_0099 exhibits an elongated C-terminus region. PFE0055c was shown to have the highest sequence identity with PFB0595w (60 %); a type II PfHsp40 which lacks the PEXEL sequence is therefore predicted to be parasite-resident; whilst PFE0055c is a PEXEL-containing type II Hsp40 that is predicted to be exported into the infected erythrocyte.

Comparison of the PFE0055c sequence with two type II PfHsp40s predicted to be exported.

The sequence alignment of PFE0055c and two type II PfHsp40s predicted to be exported is shown in figure 3.4.

```
PFA0660w        MATLRKSYVPEILYFSKFFMNACFISLLIITVNCFNYENFVCKDKGIYNEK-------IV
PFB0090c        MAIFKKYRFRENKIIFLFFIKIFLFSLFIWELCCFNKEKFQDQIQTSYYNKNNTSGNVSN
PFE0055c        MSILNKYEGKKNKI-FLFIINIILFYTLEYVLIGSNYDKHNQS----FGNEIFKNTKVFD
                *: :.*    :      *::: :: :   :   * ::. .    : ::

PFA0660w        IRYKRCLAEGNKNFFFNKDNGVFGKSSMDYYTLLGVDKGCSEDDLRRAYLKLAMKWHPDK
PFB0090c        LIIKRNLAQTQRNF-KSKNGKASTKKNEDYYSILGVSRDCTNEDIKKAYKKLAMKWHPDK
PFE0055c        FTSLRSLAE----FNSGSSRESSKTDETDYYAVLGLTKDCTQDDIKKAYRKLAMKWHPDK
                :   * **:    *   ..     ... ***::**: : *:::*:::** **********

PFA0660w        HVNKGSKVEAEEKFKNICEAYSVLSDNEKRVKYDLFGMDALKQSGFNSSNFQGNISINPL
PFB0090c        HLNAASKKEADNMFKSISEAYEVLSDEEKRDIYDKYGEEGLDKYGSNNGHSKGFKRTDPN
PFE0055c        HLNDEDKVEAERKFKLIGEAYEVLSDEEKRKNYDLFGQSGLGGTTTNDEAYYTYSNIDPN
                *:*  .* **:. ** * ***.****:***  ** :* ..*     *.         :*

PFA0660w        EVFTKAYSFYNKYFSKSSGAGNHNIFTH------------IKNLYPLRNDFSEDESSYND
PFB0090c        DVFSKFFKTETKFYSNSPSSPNGNVLFEGSLFGGSSPFSGINPRSGSGYTTSKSFSSMDK
PFE0055c        ELFSRFFSHDASSFFSQ-------GFDDFPSFQGFASMNSRRPRSSRSNIFS---RSFGR
                ::*:: :.   . : ..        : .             .        *    *

PFA0660w        VEEYEVPLYVTLEDLYNGCTKTLKVTRKRYDGCYLYYEDYFINVDIKQGWNNGTKITFHG
PFB0090c        VEEYVVPLYVTLEDLYNGTQKKLKVTRKRCQGVTTYDDEFFVTVDIKSGWCDGTTITYKG
PFE0055c        AASFEVPLQVTLEELYTGCRKKLKVTRKRFVGLNSYEDNTFITVDVKPGWSEGTKINFHG
                . .: *** ****:**.*  *.*******  *   * :: *:.**:* ** :**.*.::*

PFA0660w        EGDQSSPDSYPGDLVLVLQTKKHSKFVRKSRDLYYRHIITLEQSLTGFDFVIKSLDNRDI
PFB0090c        EGDQTSPMSNPGDLVFTIKTVDHDRFVRSYNDLIYRCPITLEQALTGHKFTIITLDNRDI
PFE0055c        EGEQSSPNEQPGDLVFIIKTKPHDRFIREGNNLIYKCYLPLDKALTGFQFSIKSLDNRDI
                **:*:** . *****: ::*  *.:*:*. .:* *:  : *:::***..* * :******

PFA0660w        HIQIDEVVKPDTKKVIKNEGMPYSRDPSIRGNLIVEFDIIYPNTIKKEQKKLIKEIFKES
PFB0090c        DIQVDEIVTPLTTRVITSEGMPYMENPKMKGNLIIEFDIIFPKKLSDEQKELIKEALGGN
PFE0055c        NVRVDDIINPNSKKIITNEGMPYSKSPSVKGDLFIEFDIVFPKKLSPEQKRTLKETLENT
                .:::*::::.* :.:::*..***** ..*.::*:*::****::*:.:. ***. :** :  .

PFA0660w        Y-
PFB0090c        GF
PFE0055c        Y-
```

Figure 3.4: Sequence alignment of the amino acid sequence of exported type II Hsp40 proteins.
The alignment was generated using ClustalW. J domains are highlighted green, G/F rich regions highlighted blue. The HPD motif is highlighted red. The KFK motif is shaded black whilst the PEXEL sequence is shaded yellow. The stars indicate identical residues while the dots indicate similar residues.

The amino acid sequence of PFE0055c was aligned with that of two other type II PfHsp40 proteins (PFA0660 and PFB0099c) exhibiting the PEXEL sequence (shaded in yellow in figure 3.4) and are exported. In addition to PFE0055c, PFA0660 and PFB0099c are

homologous to human DnaJB4 (Botha et al., 2007), a cytosolic type II Hsp40 which potentially interacts with human Hsp70. This raises the possibility that these type II Hsp40 proteins are indeed trafficked into the erythrocyte cytosol and interact with human Hsp70. A sequence identity to PFE0055c was observed between PFB0090c (43 %) and PFA0660w (39 %), both of which have been shown to be essential for the survival of *P. falciparum* (Maier et al., 2008). PFE0055c, PFB0090c and PFA0660w share lesser sequence identities in comparison with the other type II Hsp40s. The PEXEL sequence of PF11_0099 was found to be embedded within its J-domain (*data not shown*). Notably, PF11_0099 shares the lowest sequence identity to PFE0055c when compared to all PEXEL-bearing type II Hsp40s (Table 3.1). Interestingly, although PFB0595w shares the highest degree of sequence identity to PFE0055c (49 %), it lacks the PEXEL motif essential for export into the erythrocyte cytosol. The above bioinformatic analysis confirmed PFE0055c to be a type II Hsp40 protein.

Table 3.1: Percentage identities between PFE0055c and other PfHsp40s

Hsp40 protein	Percentage identity compared to PFE0055c
DnaJ	18
Sis1	22.5
PFB0595w	49
PFA0060w	40.7
PFB0090c	42
PFF1415	22.
MAL13.P1.277	9.7
PF11_0099	11
PF14_0137	13

The highest identity was between PFE0055c and PFB0595w (49 %) (Table 3.1). Since PFB0595 does not contain the PEXEL sequence, it is predicted to be parasite resident. PFA0060w and PFB0090c show relatively high sequence identity with PFE0055c (40.7 % and 42 %) respectively. Both PFA006w and PFB0090c contain the PEXEL and have been shown to be essential for *P. falciparum* survival (Maier et al., 2008).

3.2 Protein Expression and Purification

E. coli is the bacterial system that is routinely used for the expression of heterologous proteins. The advantages of using this system include the abundance of knowledge about the genetics and physiology of *E. coli*, its ease of growth, and the fact that numerous vectors have been developed in favour of maximum expression. In addition to this, considerable amounts of protein can be expressed and purified from a litre of culture in just a few days. However, it may be necessary to enhance the *E. coli* system as it has its own limitations.

P. falciparum has a genomic composition whose A/T content is more than 80 % (Gardner et al 2002) resulting in rare codon usage in *E. coli*. This codon bias has resulted in difficulties in overexpression of *P. falciparum* genes in *E. coli* (Baca and Hol, 2000). A plasmid that encodes tRNAs for rare codons, particularly arginine (R), isoleucine (I), and glysine (G) (RIG) has been engineered and shown to facilitate the heterologous production of full length *P. falciparum* proteins in *E. coli* (Baca and Hol, 2000). Matambo et al, (2004) successfully used the RIG plasmid (containing genes that encode the three tRNAs for arginine, isoleucine and glycine) to express malaria proteins.

Various strategies have been used to minimize the bias in codon usage for heterologous expression. Codon optimization consists of altering rare codons in recombinant DNA to match the codons preferred by the organism used for expression without changing the amino acids of the synthesized protein. Codon harmonization involves matching codon usage frequencies of the target gene to those that are used by the expression host (reviewed by Nicoll et al., 2006). A codon-harmonized gene has been synthesised (Bodill MSc thesis, 2009) to facilitate heterologous protein production of His_6-PFE0055c. The five-exon coding region of PFE0055c was codon-harmonized and synthesized to facilitate recombinant expression and purification of PFE0055c (Bodill MSc thesis, 2009). A *BamH*1 restriction site

was synthesized at the N-terminus of the nucleotide sequence; and *EcoR*1 and *Pst*1 sites were added to the C-terminus (Bodill MSc thesis, 2009).

E. coli host strains which contain the expressor (pQE) as well as the repressor (pREP4) plasmids can be employed for the production of recombinant proteins. Using pQE vectors, the nickel-binding 6xHis affinity tag can be placed either at the C- or N-terminus of the protein of interest. When using the 6xHis tag, anti-His antibodies can be used for detection and it does not interfere with the structure or function of the purified protein. The addition of IPTG induces the expression of recombinant proteins encoded by the pQE vectors. IPTG binds to and inactivates the *lac* repressor protein leading to sequence transcription by the host cell's RNA polymerase; and subsequent translation into the recombinant protein.

In this study, *E. coli* (M15 pRep4) cells were used for the expression and purification of PFE0055c. pRep4 is an expression vector that enhances and promotes transcription of recombinant genes inserted into multiple cloning site. pRep4 expresses the *lac* repressor protein which binds to the operator sequences and regulates recombinant protein expression. *E. coli* BL21 cells allow the expression of any gene that is under the T7 promoter, hence their use for pMSHSP expression.

3.2.1 Expression and Purification of Malaria Hsp40, PFE0055c

Confirmation of the pQE30-PFE0055c Construct

The PFE0055c coding sequence was ligated into the pQE30 backbone (Bodill, MSc thesis, 2009). The pQE30-PFE0055c construct encoding the parasitic Hsp40 PFE0055c protein was obtained from Dr E.-R. Pesce. The pQE30-PFE0055c construct was expected to produce an N-terminal 6His-tagged PFE0055c protein when transcribed and translated. The size of this

His-tagged protein was expected to be 45.7 kDa (Bodill, MSc thesis, 2009). The His-tag enables nickel affinity purification and western analysis detection of the protein using anti-His antibodies. Host cell's RNA polymerase recognizes the T5 promoter, allowing expression of the recombinant protein. The identity of the plasmid was confirmed by restriction digestion (Figure 3.5). The pQE30-PFE0055c restriction map is shown in Figure 1a, and the agarose gel electrophoresis of the digestion products in figure 1b. The size of the full-length pQE30-PFE0055c construct was expected to be 4606 bp.

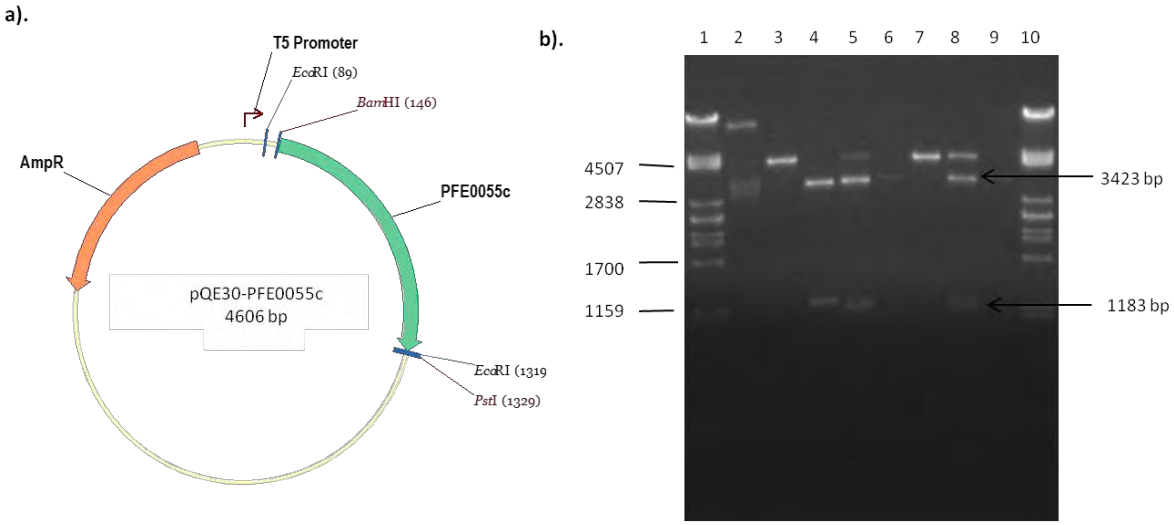

Figure 3.5: Confirmation of the pQE30-PFE0055c expression construct.
A: Plasmid map of pQE30-PFE0055c, showing restriction digestion sites *Bam*H1, *Pst*1 and *Eco*R1**. B**: Restriction digest of pQE30-PFE0055c and the parental pQE30 plasmid vector and analysis by electrophoresis using an 0.8% (w/v) agarose gel. Lane 1 and 10: *Pst*1 digestion of Lambda DNA used as the molecular mass marker. Lane 2: Uncut pQE30-PFE0055c vector. Lane 3: pQE30-PFE0055c construct digested with *Bam*H1. Lane 4: pQE30-PFE0055c construct digested with *Eco*R1. Lane 5: pQE30-PFE0055c construct digested with *Bam*H1 and *Eco*R1. Lane 6: *Bam*H1-digested pQE30 plasmid. Lane 7: *Pst*1-digested pQE30-PFE0055c construct. Lane 8: pQE30-PFE0055c construct digested with *Pst1* & *Bam*H1. Lane 9: pQE30 plasmid digested with *Pst*1.

Lane 1 (Figure 3.5b) represents the *Pst*1 restricted lambda DNA molecular mass marker. The base pair (bp) sizes of selected bands are indicated on the left. The pQE30-PFE0055c construct was linearised with restriction enzymes *Bam*H1 (Figure 3.5b; lane 3) and *Pst*1 (Figure 3.5b; lane 7); and cleaved twice with EcoR1 (Figure 3.5; lane 4) *Bam*H1/*Eco*R1

(Figure 3.5; lane 5) and *Bam*H1/*Pst*1 (Figure 3.5; lane 8). Digestion of the construct was detected by agarose gel electrophoresis (Figure 3.5b). *Eco*R1 digested at two restriction sites (89 bp and 1319 bp). *Bam*H1 and *Pst*1 digested at restriction sites 146 bp and 1329 bp respectively. A digestion with *Bam*H1 produced a single linearized DNA fragment that was larger than 4507 bp (Figure 3.5b; lane 3); and a single digestion with *Pst*1 (Figure 3.5b; lane 7) resulted in a fragment of the same size. Digestion of pQE30-PFE0055c with *Eco*R1 (Figure 3.5b; lane 4) resulted in two DNA fragments with the expected sizes of 1230 bp and 3376 bp. The larger fragment observed on lane 4 was smaller than 4507 bp but larger than 2838 bp; and the smaller fragment was larger than 1159 bp but smaller than 1700 bp, thus these fragments were estimated to resemble the expected fragments sizes. The pQE30 backbone (3423 bp) was linearized with *Bam*H1 (Figure 3.5b; lane 6) and *Pst*1 (Figure 3.5b, lane 9). The product was smaller than 4507 bp and displayed a similar mobility to the larger fragment of the pQE30-PFE0055c construct digested with *Eco*R1. This correlation would be expected since digestion of pQE30-PFE0055c with *Eco*R1 would have separated the PFE0055c coding region (1183 bp) from the pQE30 backbone. The pQE30-PFE0055c construct was subjected to double digestion with *Bam*H1 and *Eco*R1 (Figure 3.5b, lane 5) and with *Bam*H1 and *Pst*1 (Figure 3.5b, lane 8). This resulted in two DNA fragments where the larger bands corresponded to the pQE30 backbone (3423 bp). The smaller bands corresponded to the 1183 bp size of the PFE0055c coding region. The results of the restriction analysis verified the identity of the pQE30-PFE0055c construct.

Expression of recombinant His_6- PFE0055c in Escherichia coli

Protein expression studies of PFE0055c were conducted using *E. coli* M15[pREP4] cells at 37 °C. Protein expression was induced with 1mM IPTG and SDS-PAGE and western blot analyses were used to confirm the expression of His_6-PFE0055c (Figure 3.6).

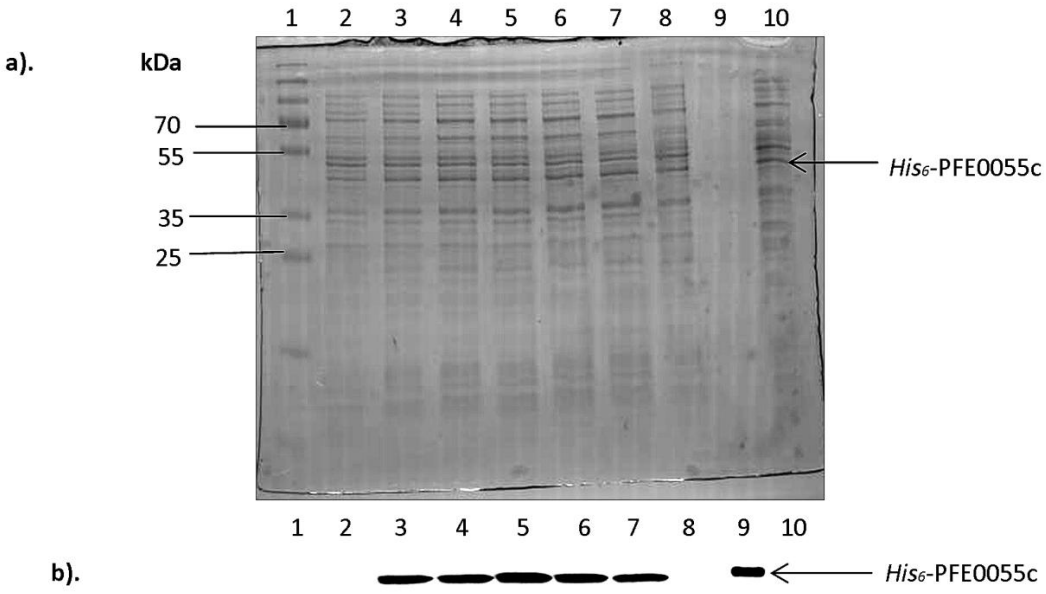

Figure 3.6: Analysis of *His*$_6$-PFE0055c protein expression in *E. coli* M15[pRep4] cells at 37 °C.

a: SDS-PAGE and **b:** Western blot analysis of samples collected during the protein induction study. The cells were grown over a period of 20 hours post induction with 1mM IPTG. Absorbance readings were taken hourly for 5 hours and overnight. For the western analysis, goat anti-PFE0055c antibody (1: 10 000 dilution) was used as the primary antibody, donkey anti-goat IgG HRP-conjugated antibody (1: 5 000 dilution) was used as the secondary antibody. Lane 1: peqGOLD molecular weight protein marker IV in kDa. Lane 2: whole cell lysate from cells transformed with pQE30-PFE0055c before induction with IPTG. Lanes 3-7 represent the 1-5 hours at which the samples were taken post induction, whilst lane 8 represents the sample collected overnight post induction. Lane 9 represents purified *His*$_6$-PFE0055c used as a positive control and lane 10 represents whole cell lysate from pQE30 transformed *E. coli* M15[pRep4] used as a negative control.

The over production of *His*$_6$-PFE0055c in *E. coli* M15[pREP4] cells is illustrated in figure 3.6. Whole cell lysated were collected at hourly intervals for a period of 20 hours post induction with 1 mM IPTG. The expected mobility (45.7 kDa) of the *His*$_6$-tagged PFE0055c protein is indicated by the arrows. The size of the protein bands on the western blot analysis were approximately 45 kDa. Although bands of expected size of the protein of interest were visible, over expression of *His*$_6$-PFE0055c in the induced samples (Figure 3.6a; lanes 3-8) was not obvious on the SDS-PAGE gel as compared to the negative control (Figure 3.6a; lane 10). The 45.7 kDa *His*$_6$-PFE0055c protein was detected between the 55 kDa and 35 kDa bands of the protein marker on the western analysis (Figure 3.6b). Expression of *His*$_6$-PFE0055c in the pre-induced sample was not detectable by the western analysis (Figure 3.6b;

lane 2); whilst at 3 hours the protein seemed to be expressed at maximum level (Figure 3.6b; lane 5). The expression of *His$_6$*-PFE0055c, however, lowered drastically over night (Figure 3.6b; lane 8). Purified *His$_6$*-PFE0055c served as a positive control and was detected by the western analysis (Figure 3.6b; lane 9). As expected, there was no protein band observed in the negative control (Figure 3.6b; lane 10). The *His$_6$*-PFE0055c protein was therefore successfully expressed at levels readily detectable by western analysis, upon induction with 1 mM IPTG in the *E. coli* M15[pREP4] cell line.

Purification of His$_6$-PFE0055c

The induction and purification conditions of PFE0055c have been optimised as part of a previous study (Bodill MSc thesis, 2009). Optimal expression temperature of 30 °C and 37 °C showed to be comparable for PFE0055c (Bodill MSc thesis, 2009). Optimal induction concentration of 0.5 mM amd 1 mM showed to be comparable for PFE0055c (Bodill MSc thesis, 2009). PFE0055c has been reported to be insoluble in *E. coli* XL1-Blue cells (Bodill MSc thesis, 2009) and thus a denaturing purification method was employed in this study. Native wash steps and elutions were employed in an attempt to refold the protein during the purification. For the purpose of the purification, *E. coli* M15[pREP4] cells were harvested at 3 hours post induction with IPTG and SDS-PAGE and western analysis were used to analyse the purification.

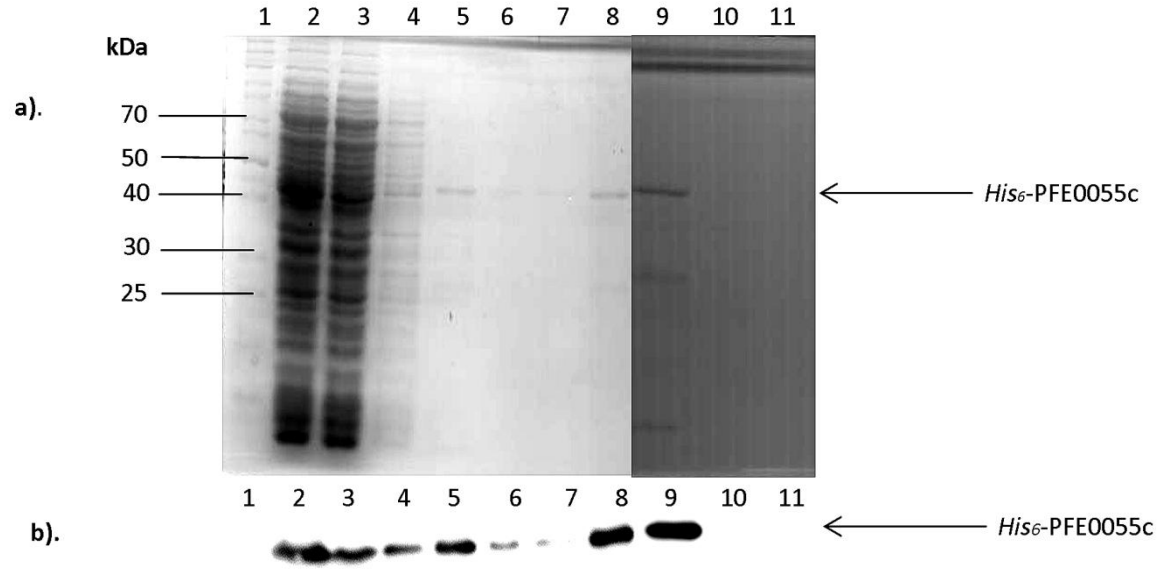

Figure 3.7: Purification of *His*$_6$-PFE0055c under denaturing conditions using nickel affinity chromatography.
A: SDS-PAGE and **b:** Western blot analysis of the purification of *His*$_6$-PFE0055c. Lane 1 represents molecular weight peqGOLD protein marker II. Lane 2 represents protein lysate after centrifugation. Lane 3 represents the unbound protein after overnight incubation with nickel-charged sepharose beads. Lane 4-8 represent washes with wash buffer (300 mM NaCl, 100 mM Tris pH 8, 10 mM Imidazole, 1 mM PMSF, and increasing concentrations of urea ranging between 6 mM and 2 mM). Lanes 9, 10 and 11 represent the first, second and third elutions (respectively). For the western analysis, goat anti-PFE0055c antibody (1: 10 000 dilution) was used as the primary antibody, donkey anti-goat IgG HRP conjugated antibody (1: 5 000 dilution) was used as the secondary antibody. These data represent a single purification, but independent purifications were performed three times with similar results.

The results of a denaturing purification of *His*$_6$-PFE0055c are illustrated in Figure 3.7. A single gel does not contain a sufficient number of wells to run more than ten samples at once, resulting in split runs of samples. The results from two gels have been displayed as a single gel to allow ease of comparison of the different samples across the two gels. Distinct bands corresponding to the expected mobility of *His*$_6$-PFE0055c are indicated by the arrow and can be seen on the SDS-PAGE (Figure 3.7a). All wash steps were performed using wash buffers containing 10 mM imidazole which resulted some elution of the *His*$_6$-PFE0055c protein. A pure preparation of *His*$_6$-PFE0055c was obtained in the first elution (Figure 3.7a; lane 9) where an elution buffer containing 1 M imidazole was used to elute *His*$_6$-PFE0055c protein. The protein bands were confirmed to be *His*$_6$-PFE0055c protein by western analysis (Figure

3.7b). *His$_6$*-PFE0055c was detected throughout the purification process, and most of the protein eluted in the final wash step (Figure 3.7, lane 8). Purified *His$_6$*-PFE0055c was detected in the first elution (Figure 3.7b, lane 9) but not in elutions 2 and 3 (Figure 3.7; lane 10 and 11). Contaminating proteins were successfully removed during the wash steps, yielding pure protein. However, proteins of a molecular weight lower than the expected molecular weight were observed. These could correspond to degradation products (Figure 3.7b; lane 9). The first elution was selected for dialysis prior to functional analyses.

3.2.2 Expression and Purification of Human Hsp70

Expression of human Hsp70A1A-His$_6$ in Escherichia coli

The pMSHSP plasmid encoding the Hsp70A1A-*His$_6$* (human Hsp70A1A with a C terminal hexahistidine tag) was supplied and verified by Ms Ingrid Cockburn. *E. coli* BL21 cells were used for protein induction studies of the human Hsp70A1A-*His$_6$* protein from the pMSHP plasmid as expression from this plasmid requires the T7 promoter system. Protein expression was induced with 1 mM IPTG and the cells were grown at 37 °C for 5 hours. The induction of Hsp70-A1A expression was assessed using SDS-PAGE and western analysis (Figure 3.8).

The expression of Hsp70A1A-*His$_6$* is illustrated in Figure 3.8. Distinct bands were observed at the expected mobility of 70 kDa for the Hsp70A1A-*His$_6$* protein on the SDS-PAGE gel, indicated by the arrow (Figure 3.8a). Although the protein did not seem to be induced when comparing samples taken before and after induction, it appeared from the western blot analysis that the maximum level of expression was reached at 3 hours and 4 hours post induction (Figure 3.8b). The presence of the protein band prior to induction (Figure 3.8b; lane 2) indicated that there was some basal expression of the Hsp70A1A-*His$_6$* protein from the pMSHSP construct, however, this band was at a lower concentration than those observed in

the lanes containing protein samples after induction (Figure 3.8b; lane 2 versus lanes 3-7). For purification purposes, *E. coli* BL21 cells were harvested 3 hours post induction at 37 °C.

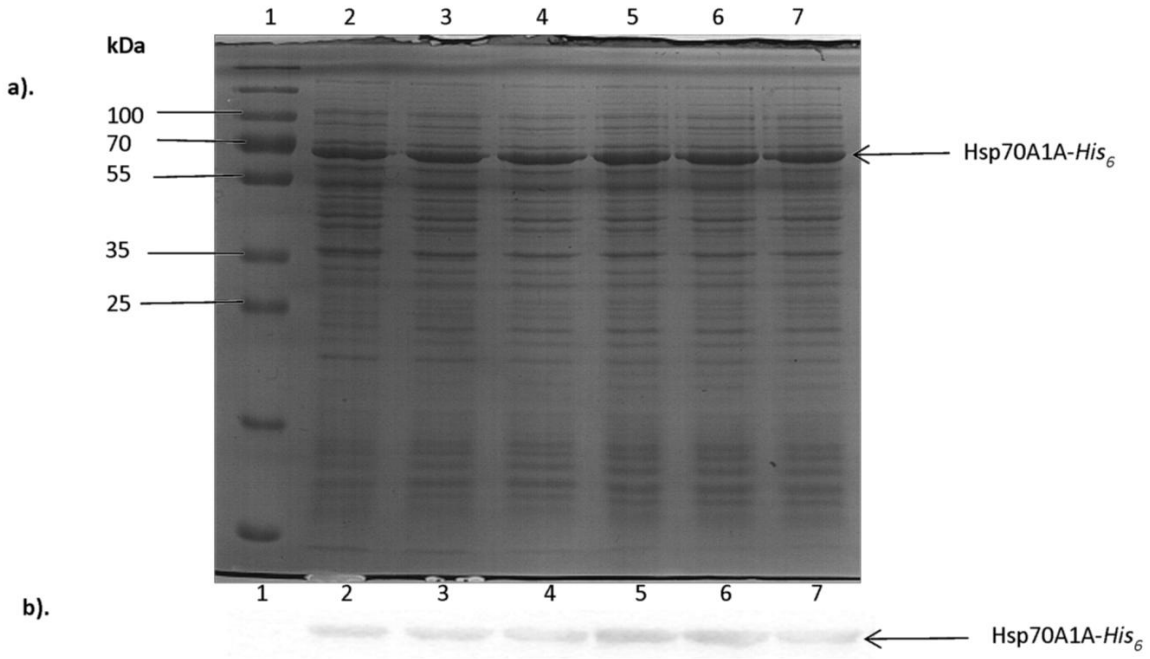

Figure 3.8: Analysis of human Hsp70A1A (pMSHSP) protein expression in *E.coli* BL21 cells
a: SDS-PAGE and **b:** Western blot analysis of expression of human Hsp70A1A protein from *E. coli* BL21 cells grown for 5 hours after induction with 1 mM IPTG. Lane 1 represents the molecular weight peqGOLD marker IV (in kDa). Lane 2 represents the total cell extract from cells transformed with pMSHSP prior to induction. Lanes 3-7 represent 1-5 hours post induction at which the samples were collected. The expected mobility (70 kDa) of the *His_6*-tagged human Hsp70A1A protein is indicated by the arrow. The size of the protein bands on the western analysis are approximately 70 kDa. For the western analysis, mouse anti-His antibody (1: 2000 dilution) was used as the primary antibody and rabbit anti-mouse (1: 2000 dilution) antibody was used as the secondary antibody.

Purification of human Hsp70A1A

Human Hsp70A1A-*His_6* was purified under native conditions from induced *E. coli* BL21 cells grown at 37 °C. Natively purified proteins are more likely to retain maximum functional activity. SDS-PAGE and western analyses were performed to assess the purification of the protein (Figure 3.9). The purification of Hsp70A1A-*His_6* is shown in Figure 3.9. Bands corresponding to the expected mobility of Hsp70A1A-*His_6* (70 kDa) are indicated by the arrow as can be seen on the SDS-PAGE (Figure 3.9a). Contaminating proteins, as seen in the

SDS-PAGE gel (Figure 3.9a), were successfully removed during wash steps with wash buffer containing 50 mM imidazole (Figure 3.9a; lanes 3-5), and finally eluted using elution buffer containing 1 M imidazole (Figure 3.9a; lanes 6-7). A band corresponding to the 6His-tagged human Hsp70A1A protein was evident throughout the purification steps, with less protein seen in the second elution (Figure 3.9a; lane 7) and almost none was observed in the third elution (Figure 3.9a; lane 8). Pure protein was eluted as judged by the presence of a single band at the correct molecular weight in the elutions, indicating a successful purification of Hsp70A1A-*His*$_6$ under native conditions. The native purification of Hsp70A1A-*His*$_6$ was further validated by western analysis (Figure 3.9b) where the presence of the Hsp70A1A-*His*$_6$ protein was confirmed (Figure 3.9b). Elutions 1-3 were selected for dialysis prior to functional analyses.

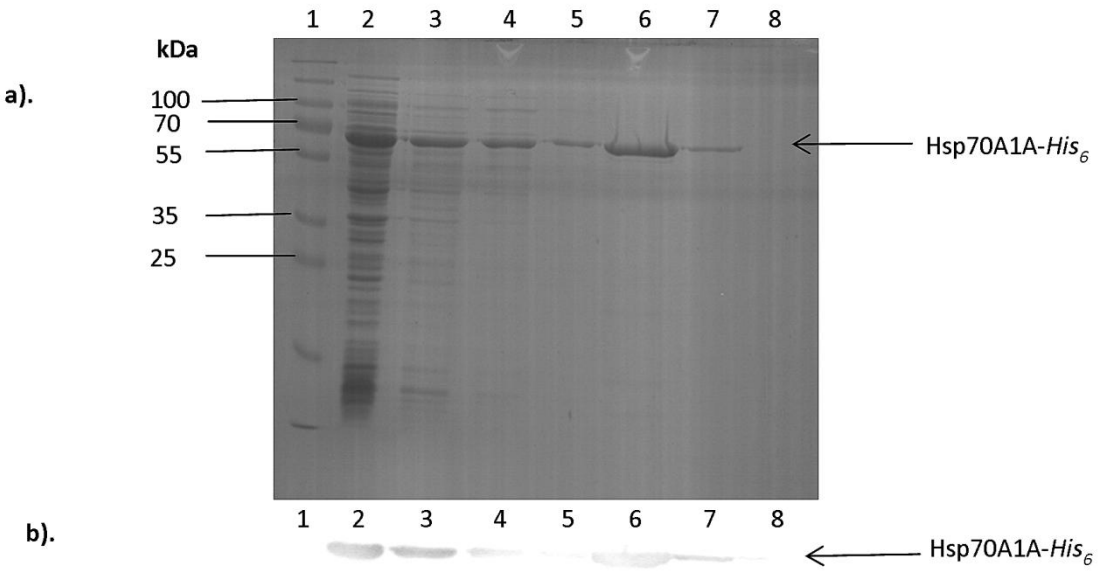

Figure 3.9: Purification of Hsp70-A1A-*His*$_6$ from *E. coli* BL21 cells using nickel chromatography.

A: SDS-PAGE and **b:** Western blot analysis of purification of Hsp70A1A-*His*$_6$- protein from *E. coli* BL21 cells. Lane 1 represents the peqGOLD molecular weight protein marker IV. Lane 2 represents the unbound protein fraction after overnight binding to nickel-charged sepharose beads. Lanes 3-5 represent the first, second and third washes respectively, with wash buffer (10 mM Tris pH 7.5, 300 mM NaCl, 50 mM Imidazole, 1 mM PMSF). Lanes 6-8 represent the first, second and third elutions respectively with elution buffer. For the western analysis, mouse anti-His antibody (1: 2000 dilution) weas used as the primary antibody and rabbit anti-mouse (1: 2000 dilution) antibody was used as the secondary antibody.

3.2.2.3 Purified Protein after Dialysis

For use in functional assays, purified proteins were dialysed to remove imidazole from the samples. Protein samples were reserved for SDS-PAGE analysis and are shown in Figure 3.10.

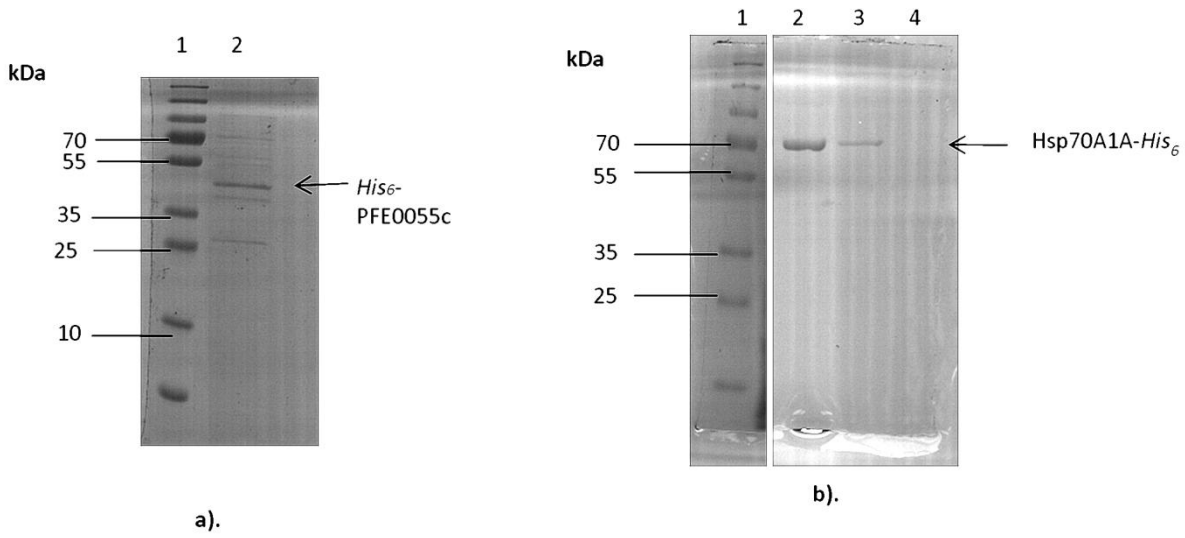

Figure 3.10: SDS-PAGE analysis of *His*$_6$-PFE0055c and Hsp70A1A-*His*$_6$ after dialysis.
a: *His*$_6$-PFE0055c after dialysis. Lane 1 represents peqGOLD molecular weight protein marker IV in kDa. Lane 2 represents elution 1 of purified protein after dialysis. **b:** Hsp70A1A-*His*$_6$ after dialysis. Lane 1 represents peqGOLD molecular weight protein marker IV in kDa. Lanes 2-4 represent elution 1-3 of purified Hsp70A1A-*His*$_6$ after dialysis.

Bands corresponding to the expected mobility of *His*$_6$-PFE0055c (45.7 kDa) and *His*$_6$-Hsp70A1A (70 kDa) were observed on the SDS-PAGE and are indicated by the arrows (Figure 3.10a and b) (respectively). This trend was observed for almost all of the purifications and dialyses performed on *His*$_6$-PFE0055c and Hsp70A1A-*His*$_6$ proteins (*data not shown*), with minor variations in protein yield as illustrated on Table 1.

3.2.3 Determination of Protein Yield

The concentration of purified protein was determined using NanoDrop (BioRAD, USA) and the yield calculated as the milligrams of purified protein per litre of bacterial culture. In three independent purifications the protein yield of *His$_6$*-PFE0055c and Hsp70A1A-*His$_6$* (Table 1) was sufficient to carry out ATPase assays. Hsp70A1A-*His$_6$* consistently yielded high levels of purified protein (0.445 mg/L^{-1}, 0.305 mg/L^{-1} and 1.342 mg/L^{-1}); whilst the yield of *His$_6$*-PFE0055c ranged between fairly low to moderate (0.13 mg/L^{-1}, 0.16 mg/L^{-1} and 0.571 mg/L^{-1}) concentrations (Table 3.2).

Table 3.2: Yield of *His$_6$*-PFE0055c and Hsp70A1A-*His$_6$* expressed in mg/L^{-1} converted to concentrations in µM.

	Purification 1		**Purification 2**		**Purification 3**	
	yield mg/L^{-1}	**Conc. µM**	**yield mg/L^{-1}**	**Conc. µM**	**yield mg/L^{-1}**	**Conc. µM**
***His$_6$*-PFE0055c**	**0.13**	**2.8446**	**0.16**	**3.501**	**0.571**	**12.495**
Hsp70a1A-*His$_6$*	**0.445**	**6.357**	**0.305**	**4.357**	**1.342**	**19.171**

These results were obtained from 3 independent purifications of each protein.

3.2.4 Western Blot Analysis for DnaK Contamination

To investigate whether there was DnaK contamination, the *His$_6$*-PFE0055c and Hsp70A1A-*His$_6$* proteins were subjected to anti-DnaK probing and analysed by western analysis.

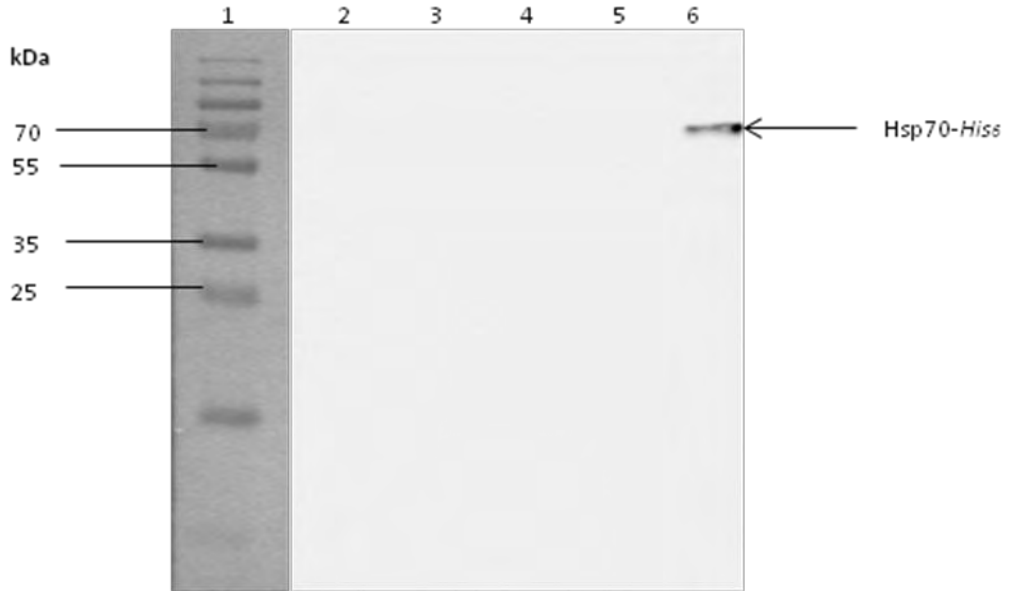

Figure 3.11: Western analysis for the presence of DnaK contamination in the *His$_6$*-PFE0055c and Hsp70A1A-*His$_6$* purification samples.
Lane 1 represents peqGold marker IV. Lanes 2-4 are the first elutions from 3 independently purified *His$_6$*-PFE0055c. Lane 5 represents previously purified *His$_6$*-PFE0055c which was a kind donation from Dr E.-R. Pesce. Lane 6 represents Hsp70A1A-*His$_6$* which was purified in this study.

As can be seen on the western blot analysis (Figure 3.11), protein bands corresponding to expected mobility of DnaK (70 kDa) were not detected in the lanes loaded with *His$_6$*-PFE0055c (Figure 3.11; lanes 2-5), but was detected in the lane containing Hsp70A1A-*His$_6$* (Figure 3.11; lane 6). Contamination with DnaK would have meant that the *His$_6$*-PFE0055c protein might have ATPase activity even in the absence of Hsp70A1A-*His$_6$*, thus would negatively affect the results. This study concluded that the purified *His$_6$*-PFE0055c was not contaminated with DnaK at levels detectable by western analysis.

3.3 Functional analysis of *His$_6$*-PFE0055c

The putative interaction between *His$_6$*-PFE0055c and Hsp70A1A-*His$_6$* was assessed using an *in vitro* ATPase assay. Hsp70 possess ATPase activity that can be stimulated above the basal rate through an interaction with a partner Hsp40 (Mayer et al., 2000). The ATPase activities of Hsp70A1A-*His$_6$* and *Medicago sativa* (alfalfa) Hsp70 were analysed in the absence and in the presence of the malarial co-chaperone *His$_6$*-PFE0055c. The alfalfa Hsp70 was included as

a positive control as this protein is commercially available and has a high ATPase activity. Other controls included *His$_6$*-PFE0055c alone. All samples were corrected for the spontaneous degradation of ATP as observed in a control experiment in the absence of chaperone proteins.

3.3.1 ATPase Assays Raw data and Calculations

The basal activities of natively purified Hsp70A1A-*His$_6$* and alfafa Hsp70 were analysed on three different occasions using three independently purified batches of protein. Co-chaperone protein *His$_6$*-PFE0055c was introduced to the reaction to assess its stimulatory ability to the enhancement of ATP hydrolysis. The results are demonstrated Figures 3.12, 3.13 and 3.14. The linear graphs illustrate the rate of ATP hydrolysis where the amount of P_i released is measured in nmol over a period of 180 minutes. Illustrated in the bar charts is the specific ATPase activity when taking into account the mg^{-1} of the proteins, expressing the final units as nmolP_i/mg/min. The raw data from three independent assays can be found below as linear curve graphs of nmol of P_i against time.

ATPase assay: replicate 1

Stimulation of the ATPase activity of Hsp701A-*His$_6$* by *His$_6$*-PFE0055c is illustrated in Figure 3.12. The nmolP_i released were measured by spectrophotometer and plotted against time (Figure 3.12a and b). The specific ATPase activity was expressed in nmolP_i/min/mg and displayed on the bar graph (Figure 3.12c) The basal specific ATPase activity of 0.4 μM Hsp701A-*His$_6$* (in the absence of the co-chaperone) was found to be 0.9 nmolP_i/min/mg (Figure 3.12c). In the reaction where *His$_6$*-PFE0055c was present; the ATPase activity of Hsp701A-*His$_6$* increased by 16 fold where the concentration of the co-chaperone was at 0.4 μM; and by 19 fold where it was at 1 μM (Figure 3.12c). Alfalfa Hsp70 was used as a control in this study and its ATPase activity was observed to be stimulated by *His$_6$*-PFE0055c. At a

concentration of 0.4 µM, *His*$_6$-PFE0055c increased the ATPase activity of alfalfa Hsp70 by 3 fold; and at a concentration of 1 µM the ATPase activity was increased by 5 fold. His6-PFE0055c proved to be more efficient at stimulating ATP hydrolysis at a concentration of 1 µM than that of 0.4 µM. However, it is worth noting that the ATPase activity of *His*$_6$-PFE0055c at concentrations of 0.4 µM and 1 µM was higher than that of Hsp70A1A-*His*$_6$ at a concentration of 0.4 µM.

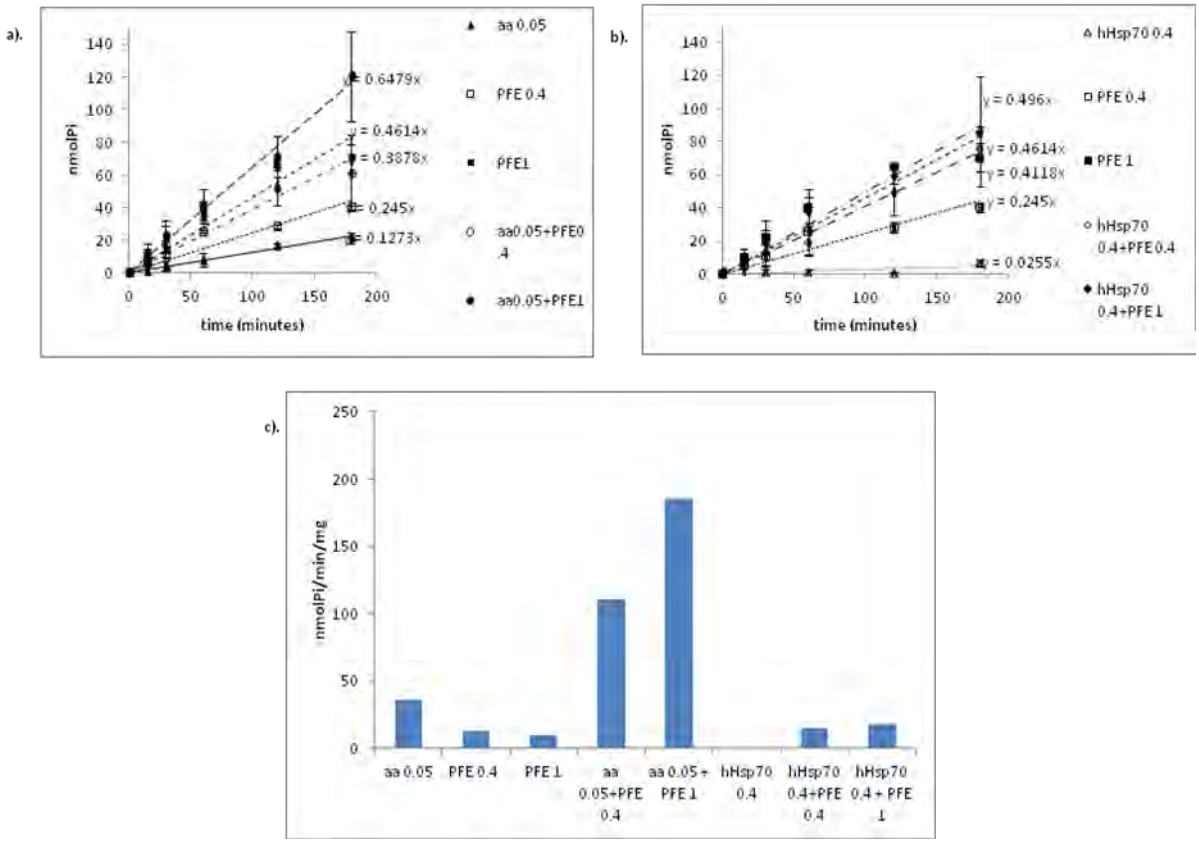

Figure 3.12 : ATPase assay raw data and calculation of specific activity (replicate 1)
The ATPase activity of alfalfa, Hsp70A1A-*His*$_6$ and *His*$_6$-PFE0055c was analysed. The linear curves (a and b) illustrate the amount of P_i in nmol, released over a period of 180 minutes. The subsequent bar chart (c) represents the specific ATPase activity (expressed in nmol Pi/min/mg) of the above mentioned proteins.

ATPase assay: replicate 2

The basal ATPase activity of 0.4 µM Hsp70A1A-*His*$_6$ was observed to be 32 fold lower than that of the same protein in the presence of *His*$_6$-PFE0055c co-chaperone when both proteins

had a concentration of 0.4 µM (Figure 3.13c). ATPase activity of Hsp70A1A-*His*$_6$ was 69 fold higher when the concentration of *His*$_6$-PFE0055c was 1 µM (Figure 3.13c).

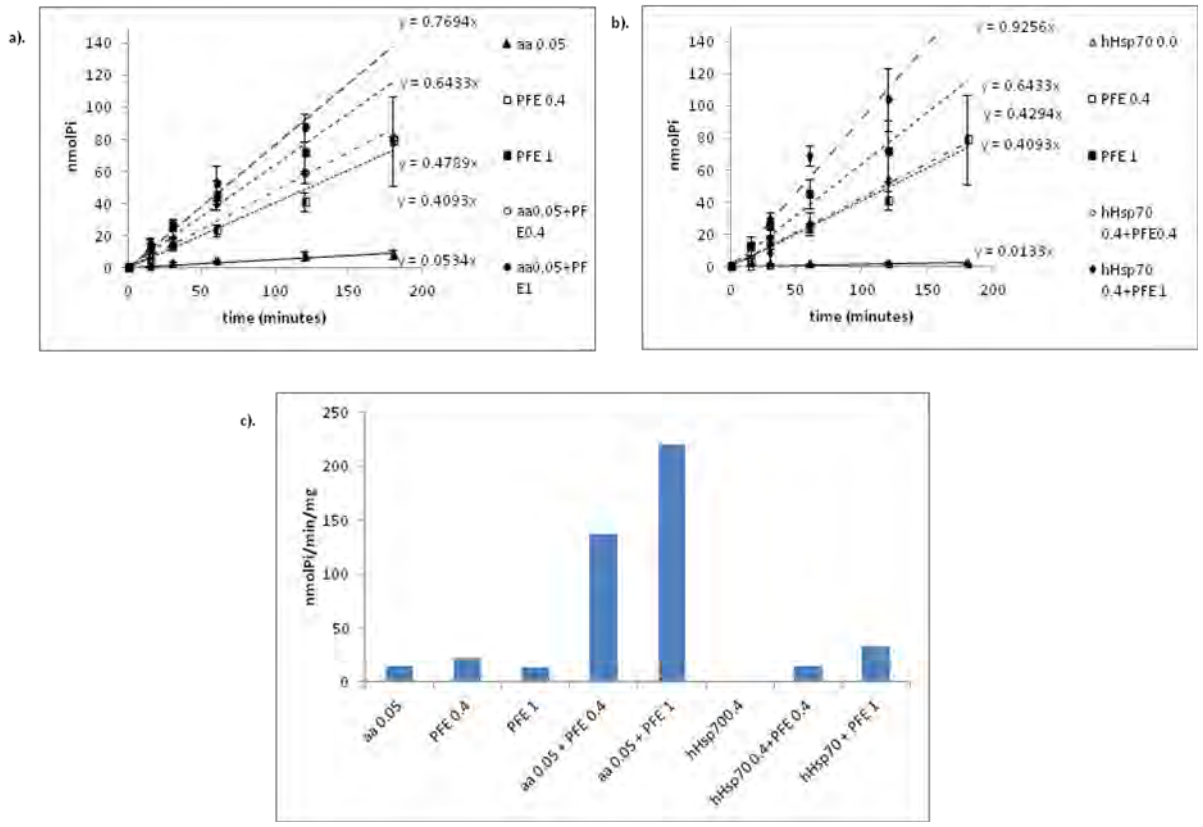

Figure 3.13 : ATPase assay raw data and calculation of specific activity (replicate 2)
The ATPase activity of alfalfa, Hsp70A1A-*His*$_6$ and *His*$_6$-PFE0055c was analysed. The linear curves (a and b) illustrate the amount of P_i in nmol, released over a period of 180 minutes. The subsequent bar chart (c) represents the specific ATPase activity (expressed in nmol Pi/min/mg) of alfalfa Hsp70, Hsp70A1A-*His*$_6$ and *His*$_6$-PFE0055c

His$_6$-PFE0055c was observed to stimulate the ATP hydrolysis of alfalfa Hsp70. At a concentration of 0.4 µM, *His*$_6$-PFE0055c increased the ATPase activity of alfalfa Hsp70 by 8.9 fold; and at a concentration of 1 µM the ATPase activity was increased by 14 fold. In the absence of chaperones, *His*$_6$-PFE0055c had a basal aATPase activity of 13.88 nmolP_i/min/mg which is higher than that of Hsp70A1A-*His*$_6$ which was 0.475 nmolP_i/min/mg.

ATPase assay: replicate 3

Stimulation of the ATPase activity of Hsp70A1A-*His_6* by *His_6*-PFE0055c is illustrated in Figure 3.14. The nmolP_i released were measured by spectrophotometer and plotted against time (Figure 4.12a and b). The specific ATPase activity was expressed in nmolP_i/min/mg and displayed on the bar graph (Figure 3.14). The ATPase activity of 0.4 µM Hsp70A1A-*His_6* in the presence of 0.4 µM *His_6*-PFE0055c was observed to be the same, whilst at a concentration of 1 µM *His_6*-PFE0055c; ATPase activity increased by 2 fold. *His_6*-PFE0055c at a concentration of 0.4 µM was observed to have a stimulating effect on the ATP hydrolysis of alfalfa Hsp70, increasing it by 2 fold. On the contrary, *His_6*-PFE0055c 1 µM decreased the ATPase activity of alfalfa Hsp70 by 3 fold.

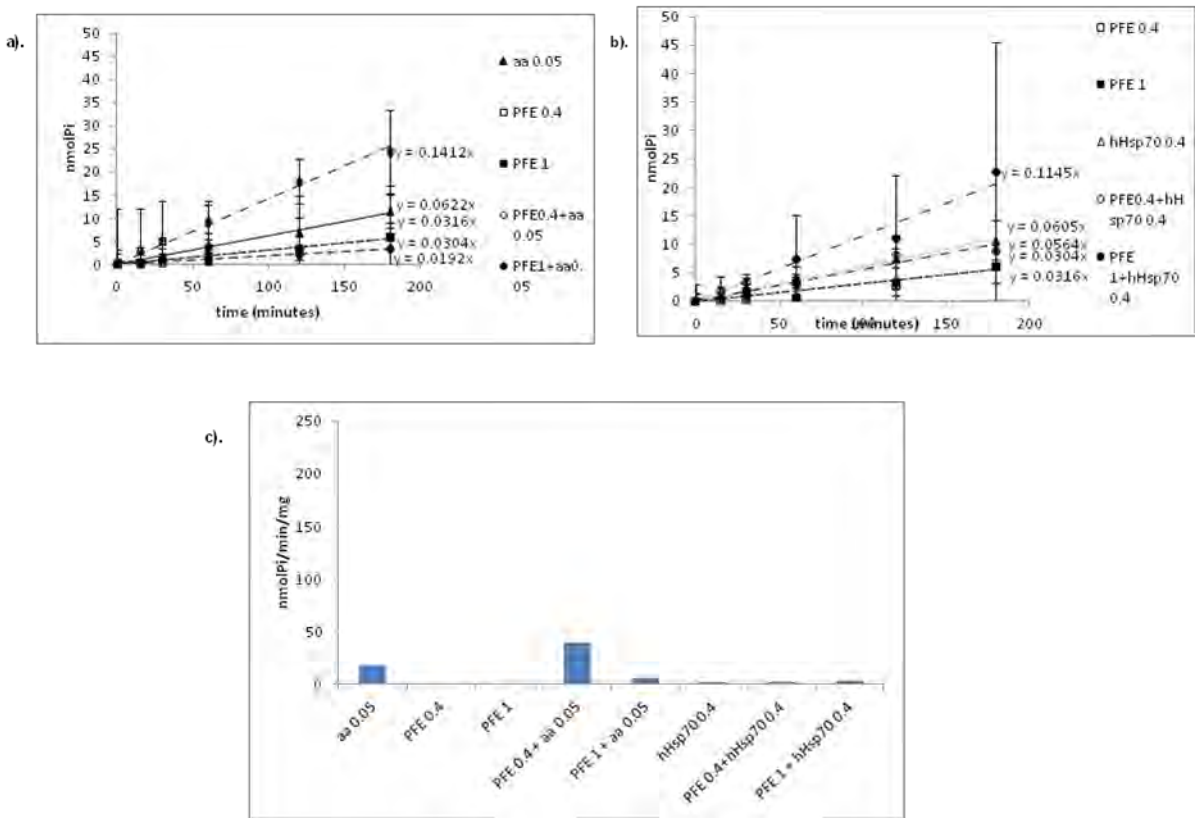

Figure 3.14 : ATPase assay raw data and calculation of specific activity (replicate 3)
The basal and co-chaperone – influenced ATPase activities of alfalfa and human Hsp70 were assessed and illustrated in graphs a and c, expressed in nmol Pi/min/mg of protein. Graphs a and b represent the amount of P_i in nmol/min.

Two of the replicate data sets (Figures 3.12 and 3.13) were consistent with each other, while the third data set (Figure 3.14) did not correlate with the other results. The average specific ATPase activity (nmolPi/min/mg Hsp70) from replicates 1 and 2 (Figures 3.12 and 3.13) was calculated and is illustrated in Figure 3.15.

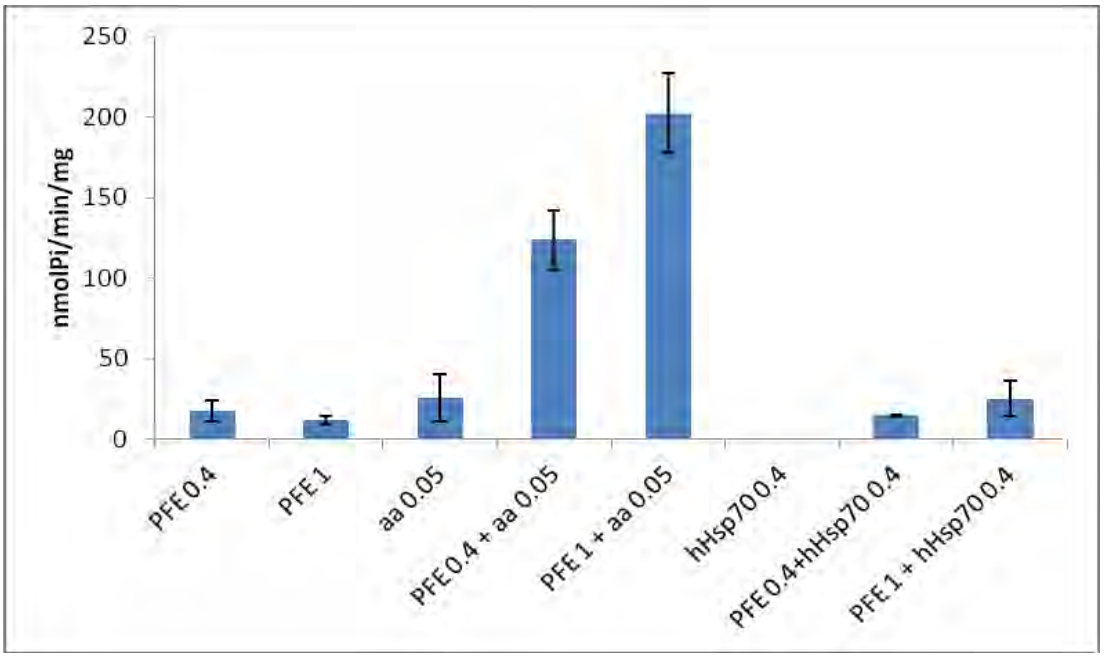

Figure 3.15: Analysis of the effect of *His*$_6$-PFE0055c on the ATPase activity of Hsp70A1A-*His*$_6$.
The above bar chart represents the average ATPase activities (represented as specific activity on nmol P_i/min/mg of protein) derived from two independent ATPase assays conducted in triplicate (Figures 3.12 and 3.13) as indicated by the respective vertical bar graphs. All data have been normalised for the spontaneous degradation of ATP. Bar 1 represents basal ATPase activity of PFE0055c at 0.4 µM. Bar 2 represents basal ATPase activity of PFE0055c at 1 µM. Bar 3 represents basal ATPase activity of alfalfa Hsp70 at 0.05 µM. Bar 4 represents ATPase activity of alfalfa Hsp70 (0.05 µM) in the presence of PFE0055c at 0.4 µM. Bar 5 represents ATPase activity of alfalfa (0.05 µM) in the presence of PFE0055c at 1 µM. Bar 6 represents basal ATPase activity of Hsp70A1A at 0.4 µM. Bar 7 represents ATPase activity of Hsp70A1A (0.4 µM) in the presence of PFE0055c at 0.4 µM. Bar 8 represents ATPase activity of Hsp70A1A (0.4 µM) in the presence of PFE0055c at 1µM. Standard errors have been indicated. Final ATPase activity is expressed in nmol P_i/min/mg of protein.

The ability of *His*$_6$-PFE0055c to stimulate the ATPase activity of human and alfalfa Hsp70 proteins was assessed (Figure 3.15). *His*$_6$-PFE0055c alone at concentrations of 0.4 and 1 µM was found to have an ATPase activity of 18.0 and 12.0 nmol P_i/min/mg respectively. Alfalfa Hsp70 was calculated to have a basal ATPase activity of 25.8 nmol P_i/min/mg at 0.05 µM. The addition of 0.4 µM and 1 µM of *His*$_6$-PFE0055c to the alfalfa Hsp70 (0.05 µM) led to an increase in the APTase activity of the chaperone to 123 nmol P_i/min/mg and 202.5 nmol

P_i/min/mg respectively. The basal ATPase activity of Hsp70A1A-*His_6* at 0.4 µM was determined to be 0.64 nmol P_i/min/mg. Incubation of Hsp70A1A-*His_6* in the presence of *His_6*-PFE0055c at 0.4 µM and 1 µM resulted in a recorded ATPase activity of 15.0 nmol P_i/min/mg and 25.4 nmol P_i/min/mg respectively.

ATPase activity was stimulated in the presence of *His_6*-PFE0055c and alfalfa Hsp70. However, it was not possible to ascertain whether the stimulation was due to *His_6*-PFE0055c stimulating alfalfa Hsp70, or vice versa. The ATPase activity of alfalfa Hsp70 and *His_6*-PFE0055c together was observed to be higher at a greater dose (1 µM) of the co-chaperone (123.8 nmol P_i/min/mg and 202.5 nmol P_i/min/mg respectively) (Figure 3.15), when compared to the ATPase activity in the absence of *His_6*-PFE0055c (25.8 nmol P_i/min/mg). This potentially implied that *His_6*-PFE0055c may have a stimulating effect on the ATPase activity of alfalfa Hsp70. *His_6*-PFE0055c at 1 µM was able to stimulate the ATPase activity of Hsp70A1A-*His_6* (25.4 nmol P_i/min/mg) when compared to *His_6*-PFE0055c at 0.4 µM on Hsp70A1A-*His_6* (15.02143 nmol P_i/min/mg) (Figure 3.15). This dose-dependent stimulatory trend was observed across two replicates.

Of great concern, however, was the fact that *His_6*-PFE0055c (18.0 nmol P_i/min/mg at 0.4 µM and 12.0 nmol P_i/min/mg at 1 µM) displayed ATPase activity and that this activity was greater than that of Hsp70A1A-*His_6* (0.64 nmol P_i/min/mg at 0.4 µM) (Figure 3.15). The possibility of DnaK contamination was excluded by means of a western analysis using anti-DnaK antibodies.

Chapter 4 : Discussion

4.1 Discussion

The main objective of this study was to investigate co-chaperone properties of PFE0055c, a *P. falciparum* type II Hsp40 chaperone exported to the cytosol of infected erythrocytes. The construct was obtained as previously discussed (section 3.2) and successfully expressed for subsequent use in functional assays. Protein yields sufficient for ATPase assay were successfully obtained (Table 3.1)

4.1.1 Bioinformatic Analysis of PFE0055c

All Hsp40 proteins that were aligned in this study comprised the J-domain (highlighted green) which contains the highly conserved HPD motif (highlighted red). In this study, PFA0660w, PFB0090c, PFE0055c and PF11_0099 were found to contain the PEXEL motif as consistent with (Botha et al., 2007); although it is questionable as to whether PF11_0099 contains a true PEXEL motif since it is positioned within the J-domain. PFE0055c contains the conserved KFK motif, consistent with the study by (Hennessy et al., 2000). DnaJ contains a G/F-rich region (highlighted blue) with the conserved DIF motif (Cajo et al., 2006) whilst PFE0055c lacks the DIF motif. PFE0055c was not found to contain any cysteine repeats as can be seen from DnaJ, a type I Hsp40. This study showed that type II Hsp40 proteins are closely related to one another according to their class as shown by (Hennessy at al., 2000). Due to the similarity of PFE0055c to other type II Hsp40s, PFE0055c can be classified as a type II Hsp40 protein. PFE0055c, PFA0660w and PFB0099c are exported type II Hsp40 proteins and they are similar to the human DNAJB4, which interacts with human Hsp70 (Botha et al., 2007). If *P. falciparum* exports type II Hsp40 into the host cytosol, it may be proposed that they perform a similar function of interacting with human Hsp70, making them possible drug targets.

4.1.2 Production and Purification of Malaria Hsp40, PFE0055c

Recombinant *His*$_6$-PFE0055c was observed at the expected band of 46 kDa upon expression, and was successfully purified under denaturing conditions by nickel affinity chromatography. Our results support those of previous studies (Matambo et al., 2004; Ramya et al., 2006) which showed that proteins successfully purified by denaturing purification could regain their functional activity once the denaturant was removed. The native state of the protein may not always be achieved however, despite removal of the denaturant (Kathir et al., 2005) and functional activity of protein could therefore be compromised. Additional analyses should have been included in order to confirm that the protein was in fact refolded. The refolding of *His*$_6$-PFE0055c could have been confirmed using spectrofluorimetry (to rule out the presence of aggregates) or fourier transform infrared spectroscopy (FTIR) to confirm the secondary structure of the protein. However, an alternative strategy could be to try to purify the protein under native conditions. An example of such a strategy that would allow this would be codon optimisation. Codon optimisation has successfully been performed for another malarial Hsp40, Pfj4, where high levels of expression were obtained in comparison with the expression of rare tRNA and co-expression of PfHsp70 (Nicoll et al., 2007). Codon-optimization may be beneficial in that the coding region may be re-designed to include the optimal codons, particularly those frequently used by the host organism. This will result in the improvement of the rate of protein synthesis and subsequent larger protein yields. However, it may be disadvantageous in that the high rates of protein synthesis may lead to protein misfolding. Codon-optimisation could be employed as an alternative approach for future expression studies of *His*$_6$-PFE0055c.

4.1.3 Protein Expression and Purification of Human Hsp70A1A-*His_6*

Human Hsp70A1A was successfully expressed and purified fron *E. coli* BL21 cells without the RIG plasmid as prescribed in previous studies (Matambo et al., 2004). Hsp70A1A was significantly over-produced and purified under native conditions. Hsp70A1A was observed at the expected band of 70 kDa. The basal ATPase activity of human Hsp70A1A was determined to be 0.6 nmolP_i/min/mg. Previous reports have determined the ATPase activity of human Hsp70 to be 2 nmolP_i/min/mg (Bimston et al., 1998), 0.5 nmolP_i/min/mg (Chamberlain and Burgoyne, 1997) and 5.5 nmolP_i/min/mg (Olson et al., 1994). Different ATPase assays were used in these studies and it is possible that this may account for the difference. Bimston and colleagues used a radioactive assay for ATPase activity, while the Olsen and Chamberlain groups used colorimetric assays similar to those used by us. However, none of these studies report the specific isoform of Hsp70 used in ATPase activity assays. Therefore, it is possible that the differences in activity may be as a result of different isoforms being used. Our data are however consistent with the findings of Chamberlain and colleagues (Chamberlain and Burgoyne, 1997).

4.1.4 Effect of His_6-PFE0055c on the ATPase Activity of Hsp70 from different species

Hsp40s function to regulate ATP-dependent polypeptide binding by Hsp70s (Lieberek et al., 1991). Since Hsp40 proteins localise to different sites within the cell, they are able to form different interactions with Hsp70s and bind to unique clients at these sites. Multiple members of the Hsp40 family can interact with a single Hsp70 protein, although the mechanism by which different Hsp40s interact with Hsp70s is not yet fully defined; and neither is the mechanism by which Hsp40s function to bind non-native polypeptides. ATP hydrolysis leads to a conformational change in Hsp70 that stabilizes Hsp70-polypeptide complexes. Nucleotide exchange factors (NEFs) facilitate the release of Hsp70-bound ADP, decreasing

Hsp70 affinity for substrate. A nucleotide exchange factor, GrpE in *E. coli*, regulates substrate release from Hsp70 by exchanging ADP for ATP (Lieberek et al., 1991). In mammals, Bcl-2-associated athanogene (BAG-1) facilitates this nucleotide exchange (Höhfeld and Jentsch, 1997). The *Plasmodium falciparum* elongation factor 1beta (PfEF-1β) is present throughout the parasite's red blood cell stages (Mamoun et al., 1998), although its expression up-regulated at the early schizont stage of the life-cycle (Mamoun and Goldberg 2001). PfEF-1β has not been shown to be exported, suggesting that BAG-1 could be used as the NEF in Hsp40-Hsp70-polypeptide interactions in the cytosol of infected erythrocytes. Not only do Hsp40 proteins deliver specific clients to Hsp70 through peptide binding domains (Cyr et al., 1994; Cheetham and Caplan 1998), they also stabilise Hsp70-polypeptide complexes (Liberek et al., 1991). The formation of Hsp40-Hsp70-polypeptide complexes is thought to be important intermediate in the Hsp70 polypeptide-binding and release cycle and appears to facilitate substrate transfer from Hsp40 to Hsp70 (Han and Christen 2003). Hsp40s contain an HPD motif in the loop region of the J-domain. Mutations in the HPD motif hinders ATPase activity regulation of Hsp70 by Hsp40 (Tsai and Douglas 1996; Mayer et al., 1999) suggesting that the HPD motif is essential for this role. The J-domain's ability to stimulate ATPase activity of Hsp70 is enhanced by the presence of peptides bound to the polypeptide-binding site of Hsp70 (Bukau and Horwich, 1998), however, the J-domain alone has been shown to be sufficient to stimulate Hsp70 ATPase activity (Wall et a. 1994).

The aim of this study was to investigate any possible co-chaperone activity of His_6-PFE0055c. This was performed by stimulating ATP hydrolysis in the presence of the His_6-PFE0055c and two Hsp70 (human Hsp70A1A and alfalfa Hsp70) and measuring the release of inorganic phosphate using a colorimetric assay. The ATPase assay used in this study measured the steady stage ATPase activity, which measures both the hydrolysis and nucleotide exchange stages of the Hsp70 cycle. Therefore, it is possible that the Hsp40 stimulation of ATPase activity using this assay may be as a result of effects on both ATP

hydrolysis and nucleotide exchange rates of the Hsp70. This is in comparison to single turnover, radioactive assays used by Botha and colleagues which only measured the hydrolysis of ATP (Botha et. al., 2011). Using the single turnover assay would permit the determination of change of rate of ATP hydrolysis alone. However, despite this and being less sensitive than radioactive assays, the use of colorimetric single turnover assay is established in chaperone studies (Chamberlain and Burgoyne, 1997).

In this study, *His*$_6$-PFE0055c was successfully purified and shown to have a basal ATPase activity at least 11-fold higher than that of Hsp70A1A-*His*$_6$. To date, no work has been carried out on the ATPase activity of PFE0055c, therefore, the nature of its activity is not known. PFE0055c was exported into the infected erythrocyte cytoplasm (Bhattacharjee et al., 2008) where it is said to localize "J-dots" (Kulzer et al., 2010) and not Maurer's clefts as previously determined (Bhattacharjee et al., 2008). The high levels of ATPase activity with the *His*$_6$-PFE0055c alone could have suggested that this protein had ATPase activity. To date there have been no reports of any of the hundred of identified Hsp40 proteins with ATPase activity. Therefore, it would seem unlikely that *His*$_6$-PFE0055c exhibited endogenous ATPase activity.

The most likely explanation for the ATPase activity is due to a contaiminating protein, such as the bacterial Hsp70 DnaK. In *E. coli*, DnaK plays a role in many cellular functions including the refolding of denatured proteins (Mayer et al., 2000, Hartl and Hayer-Hartl, 2002). A reaction cycle of ATP binding, hydrolysis and nucleotide exchange controls the chaperone activity of the Hsp70 family (Figure 1.4). The ATP hydrolysis of ATP to ADP is accelerated by the interaction of the DnaJ J-domain with DnaK. The presence of contaminating DnaK in recombinant proteins produced in *E.coli* thus may contribute to elevated ATPase activity, thereby making the exclusion of possible DnaK contamination a vital part of every experiment. However, *His*$_6$-PFE0055c yielded from this study was shown

to be free of *E. coli* Hsp70 (DnaK) contamination by Western analysis. Despite the fact that Western analysis is a sensitive and specific technique to identify contaminating proteins, it is possible that some DnaK was still present in the purification. DnaK contamination can be overcome by including additional ATP washes to remove non-specifically bound DnaK. Alternatively, protein expression could be performed using host cells that do not contain DnaK, such as *E.coli* strain BB1994. Alternatively, the ATPase activity may have been due to contamination with another non-chaperone ATPase which could have been removed by performing an additional purification step, such as gel exclusion or ion exchange chromatography.

When the effect of *His_6*-PFE0055c on the ATPase activity of the Hsp70A1A-*His_6* was analysed, it was not clear whether or not PFE0055c was able to stimulate Hsp70A1A. There was a dose-dependent increase in the ATPase activity when the two proteins were incubated together, but this was determined to be an additive effect. The ATPase activity of the two proteins together was equivalent to the sum of the individual ATPase activities. Therefore, it is unlikely that PFE0055c was able to stimulate the ATPase activity of Hsp70A1A. These results may be due to the fact that PFE0055c exhibited basal ATPase activity which masked the stimulation of the Hsp70A1A ATPase activity. Interactions between molecular chaperones are thought to be vital for the survival of RBC due to the ability to refold misfolded proteins. Proteomic studies have identified a number of human Hsp70 isoforms (Hsp70A1A, Hsp70A4, Hsp70A8) along with certain other chaperones (Hsp90α and stress inducible phosphoprotein 1/ STIP1). Only a single human Hsp40, DNAJC5, has been identified from human RBC proteomic studies (Goodman etal., 2007, D'Alessandro et. al., 2010).

Our study further showed that *His_6*-PFE0055c was able to interact with alfalfa Hsp70. Alfalfa Hsp70 had a stimulated activity of up to 8 fold in the presence of *His_6*-PFE0055c as opposed to the ATPase activity in the absence of *His_6*-PFE0055c. This suggested that PFE0055c was

interacting with alfalfa Hsp70 to stimulate ATP hydrolysis. The ATPase activity of alfalfa was much greater than Hsp70A1A and therefore the stimulation was observable above the background ATPase activity of PFE0055c. Additional bioinformatics analysis of Pf-Hsp70-1 and Hsp70A1A with alfalfa Hsp70 indicated a high degree of sequence identity at the amino acid level (*data not shown*). PfHsp70 was more similar to alfalfa Hsp70 (76% identical) than to human Hsp70A1A (73% identical).

It would have been interesting to explore the interactions between His_6-PFE0055c and PfHsp70. PfHsp70s do not contain a PEXEL sequence although PfHsp70-1 and PfHsp70-3 have been located outside of the PV (Nyalwidhe and Lingelbach, 2006), suggesting that they play role there. Studies reveal that a type I PfHsp40 chaperone (PF14_0359) stimulates the rate of ATP hydrolysis of the cytosolic PfHsp70 (Hsp70-1) and that of human Hsp70A1A (Botha et al., 2011). Furthermore, it was found that the cytosolic PF14_0359 is upregulated under heat shock conditions in similar fashion to that of Hsp70-1 (Botha et al., 2011). PF14_0359 does not contain a PEXEL motif (Botha et al., 2007) and was not exported. However, it has been shown to stimulate the ATP hydrolysis rate of both PfHsp70-1 and human Hsp70A1A (Botha et al., 2011). These data suggest that there is a formation of PfHsp70-PfHsp40 partnerships in the malaria parasite; furthermore, exported PfHsp40s potentially play a role in the stimulation of host Hsp70s *in vivo*. However, the purification of PfHsp70 was not successful in this study and thus such experiments should be prioritised for future studies.

4.2 Future Work

Future work involves purifying contaminant-free PFE0055c to further characterise the protein. Recent studies localise PFE0055c to “J-dots”; however, further experiments are still to be done to conclude whether or not there is an interaction between PFE0055c and Hsp70A1A. Alternatives to study the interaction between the two proteins could include cell biological approaches such as immunoprecipitaion from parasite-infected erythrocytes or biophysical interaction studies such as surface plasmon resonance (SPR) using purified proteins. Further experiments might also include using an assay that is independent of ATPase activity, such as the aggregation suppression assay using thermally denatured MDH or chaperone refolding assays. In addition, the co-localisation of the two proteins could be studied in the parasite by confocal microscopy.

Chapter 5 : References

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