MOLECULAR CHARACTERISATION OF THE CHAPERONE PROPERTIES OF *PLASMODIUM FALCIPARUM* HEAT SHOCK PROTEIN 70

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Abstract

Heat shock protein 70 (called DnaK in prokaryotes) is one of the most prominent groups of chaperones whose role is to prevent and reverse protein misfolding. PfHsp70 is a heat-inducible cytoplasm/nuclear localised *Plasmodium falciparum* Hsp70. PfHsp70 is thought to confer chaperone cytoprotection to *P. falciparum* during the development of malaria fever. The objective of this study was to examine the chaperone properties of PfHsp70 using a bioinformatics approach, coupled to *in vivo* and *in vitro* analysis. Structural motifs that qualify PfHsp70 as a typical Hsp70 chaperone were identified. Although PfHsp70 has a higher similarity to human Hsc70 than *E. coli* DnaK, *in vivo* complementation assays showed that PfHsp70 was able to reverse the thermosensitivity of *E. coli dnaK756* (a temperature sensitive strain whose DnaK is functionally compromised). Two residues (V401 and Q402) in the linker region of PfHsp70 that are critical for its *in vivo* function were identified. Constructs were generated that encoded the ATPase domain of PfHsp70 and the peptide binding domain of *E. coli* DnaK (to generate PfK chimera); and the ATPase domain of *E. coli* DnaK fused to the peptide binding domain of PfHsp70 (KPf). The two chimeras were tested for their ability to reverse the thermosensitivity of *E. coli dnaK756* cells. Whilst KPf was able to reverse the thermosensitivity of the *E. coli dnaK756* cells, PfK could not. Previously, PfHsp70 purification involved urea denaturation. Using a detergent, polyethylenimine (PEI), PfHsp70 was natively purified. Natively purified PfHsp70 had a basal ATPase activity approximately two times higher than the previously reported activity for the protein purified through urea denaturation. PfJ4, a type II Hsp40, could not stimulate the ATPase activity of PfHsp70 *in vitro*. Arch and hydrophobic pocket substitutions (A419Y, Y444A

and V451F) were introduced in the PfHsp70 peptide binding domain. Similar substitutions were also introduced in the KPf chimera. PfHsp70-V451F (hydrophobic pocket mutant) had marginally compromised *in vivo* function. However, a similar mutation (V436F), introduced in KPf abrogated the *in vivo* function of this chimera. The arch and hydrophobic pocket derivatives of PfHsp70 exhibited marginally compromised *in vivo* function, whilst equivalent mutations in KPf did not affect its *in vivo* function. The ability of PfHsp70 and its arch/hydrophobic pocket mutants to suppress the heat-induced aggregation of malate dehydrogenase (MDH) *in vitro* was investigated. Whilst PfHsp70 arch mutants displayed marginal functional loss *in vivo*, data from *in vitro* studies revealed that their functional deficiencies were more severe. This is the first study in which an Hsp70 from a parasitic eukaryote was able to suppress the thermosensitivity of an *E. coli* DnaK mutant strain. Findings from the *in vivo* and *in vitro* assays conducted on PfHsp70 suggest that this protein plays a key role in the life-cycle of *P. falciparum*. Furthermore, this study raised insights that are pertinent to the current dogma on the Hsp70 mechanism of action.

Declaration

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

_____

_____

This _____ day of _____ 2007

Dedication

This thesis is dedicated to

My wife, Venencia and our daughters, Tariro and Rutendo.

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Except the LORD build the house, they labor in vain that build it: except the LORD keep the city, the watchman waketh but in vain. Psalms 127[1]. Let all glory be to God!

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List of Symbols

1. Abbreviations of units

Symbol	Interpretation
%	Percent
α	alpha
β	beta
λ	lambda
µl	microlitre
A_{360}	Absorbance at 260 nanometers
A_{600}	Absorbance at 600 nanometres
bp	base pair
kDa	kiloDalton
µM	micromolar
nmol	nanomoles
pmol	picomoles
nm	nanometres
^{o}C	degree Celsius
ml	milliliter
w/v	weight per volume
v/v	volume per volume

2. IUPAC amino acid single letter codes (SLC), and corresponding DNA codons

Amino Acid	SLC	DNA codons
Isoleucine	I	ATT, ATC, ATA
Leucine	L	CTT, CTC, CTA, CTG, TTA, TTG
Valine	V	GTT, GTC, GTA, GTG
Phenylalanine	F	TTT, TTC
Methionine	M	ATG
Cysteine	C	TGT, TGC
Alanine	A	GCT, GCC, GCA, GCG
Glycine	G	GGT, GGC, GGA, GGG
Proline	P	CCT, CCC, CCA, CCG
Threonine	T	ACT, ACC, ACA, ACG
Serine	S	TCT, TCC, TCA, TCG, AGT, AGC
Tyrosine	Y	TAT, TAC
Tryptophan	W	TGG
Glutamine	Q	CAA, CAG
Asparagine	N	AAT, AAC
Histidine	H	CAT, CAC
Glutamic acid	E	GAA, GAG
Aspartic acid	D	GAT, GAC
Lysine	K	AAA, AAG
Arginine	R	CGT, CGC, CGA, CGG, AGA, AGG
Stop codons	Stop	TAA, TAG, TGA

List of outputs

Publications

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Chapter One

Introduction

1.1 Protein folding, misfolding and aggregation

Interest in the study of protein folding goes as far back as 1931 when Wu observed the denaturation of a protein and its reversion to the native state. In 1959, Kauzman proposed that hydrophobicity was at the centre of protein folding. However, direct evidence suggesting that the primary amino acid sequence of a protein dictates its folding pattern *in vitro* was only observed later (Anfinsen, 1973). In obedience to Anfinsen's observation, some denatured proteins can spontaneously refold into the native form in milliseconds *in vitro*. However, spontaneous folding occurs only if the energy status and size of the protein permits (Dinner *et al.*, 2000), hence practically, proteins tend to aggregate and misfold. Furthermore, the cellular environment poses huge obstacles to the protein folding process because of its highly crowded nature (Zimmerman and Trach, 1991).

Aggregation of proteins is exacerbated by the fact that the attainment of a functional, stable tertiary structure of a protein demands the availability of a complete domain (100-300 amino acids) (Young *et al.*, 2004). Because of this, translating peptides often appear in non-native conformations as they emerge from the ribosomes until adequate structural features are made available. In addition, during translocation, part of a peptide may get exposed to conditions that favour folding whilst the rest of the protein is in an environment that disfavours folding (Rothman and Kornberg, 1986). Consequently, in practice, proteins tend to misfold and aggregate and this has repercussions on cell physiology since half times for aggregated proteins to reorganise themselves into functional forms are too long to meet physiological demands (Slepenkov and Witt, 2002). Besides the folding challenges that proteins have to face at the post-transcriptional stages, physiological stresses (such as pH changes, heat, ischaemia, heavy metals) promote denaturation of proteins (Somero, 1995). Therefore, proteins constitute the most prominent molecular victims of cellular stress.

Under stressful conditions, many proteins occur in a state of equilibrium between the native and partially unfolded state. Aggregation amongst the same protein molecules may follow (resulting in the formation of homo-aggregates), or could involve different protein species

associating (generating heteroaggregates) (Ben-Zvi and Goloubinoff, 2002). The fate of a partially unfolded protein depends on the prevailing conditions in the cell. The presence, even in minute concentrations, of some protein molecules in the cell with a propensity to aggregate could trigger chain aggregation reactions between those peptides and other species of partially unfolded proteins in the vicinity (Ben-Zvi and Goloubinoff, 2002).

Excessive aggregation of proteins can result in a number of pathological conditions, which are characterised by deposition of amyloid fibrils in the respective organs (Dobson, 2004). Some of the pathological conditions are hypercholesterolaemia, cystic fibrosis, Huntington's disease, Alzheimer's diseases, and Parkinson's disease (Dobson, 2004). Amyloid fibrils are products of highly organised polymers of unfolded protein (Dobson, 2004). Controversy abounds as to whether it is the mature fibrils or their soluble oligomers that initiate toxicity in cells (Bucciantini *et al.*, 2002). An investigation by Novitskaya *et al.* (2006) suggested that both mature fibrils and their soluble oligomeric precursors are cytotoxic.

1.2 Molecular chaperones

1.2.1 The role of molecular chaperones

In order to avert and reverse protein misfolding and aggregation, the cell has developed amongst other responses, a molecular chaperone-mediated system. Molecular chaperones are proteins that facilitate the non-covalent organisation of other proteins into their functional three-dimensional structures without becoming permanent components of the products themselves (Ellis, 1987). Therefore a molecular chaperone recognises, binds and facilitates the proper folding of an otherwise misfolded protein (Hendrick and Hartl, 1993). Feder and Hofmann (1999) summarised the association between a chaperone and its protein substrate as responsible for: (a) keeping the partner protein in a folding-competent, folded or unfolded state, (b) facilitating localisation, import and/or export of target protein, (c) reducing the degree of aggregation of non-native proteins and (d) facilitating the degradation of non-native and aggregated proteins. As evidence for their role in protein folding, some chaperones associate with newly synthesised proteins exiting the ribosomes, while others have demonstrated *in vitro* refolding capability (Eggers *et al.*, 1997; Hartl and Hayer-Hart, 2002).

Whilst molecular chaperones can manage protein aggregation and misfolding during mild stress conditions, they do so with limited success during extreme cellular stresses. Therefore, the level of accumulated misfolded and aggregated protein overwhelms the chaperone response at some point. For example, up to 30% of protein constitutes defective ribosomal products (newly synthesised proteins that never attain the native state) and the cell requires a fast and efficient way of degrading these misfolded products (Schubert *et al.*, 2000). In eukaryotes, the ubiquitin-proteasome system is employed in the degradation of malfolded proteins (Wolf and Hilt, 2004). Chaperones bind to misfolded protein, thereby stabilising them to facilitate refolding or degradation. Co-chaperones associated with the resulting protein complex determine the fate of the peptide substrate in the complex. For example, co-chaperones that link chaperone systems with the ubiquitin-proteasome system have been identified (Höhfeld *et al.*, 2001; Esser *et al.*, 2004). An example of these co-chaperones is C-terminal Hsp70 interacting protein (CHIP), which can interact with Hsp70 and Hsp90 as well as the ubiquitin-proteasome system (Connell *et al.*, 2001; Jiang *et al.*, 2001). A close partnership between the human neuronal cell enriched Hsp40 co-chaperone, Hsj1 and CHIP in the degradation pathway has been established (Westhoff *et al.*, 2005). Another co-chaperone that has been implicated in the degradation process is Bcl-2-associated athanogene (Bag-1) which serves as a nucleotide exchange factor (NEF) of Hsp70 and at the same time links the chaperone to the proteasome through a motif that recognises the ubiquitin based protein degradation system (Lüders *et al.*, 2000).

1.2.2 Functional specialisation of molecular chaperones

Although highly conserved, molecular chaperones are specialised and functionally compartmentalised (McMillan *et al.*, 1994). In general, chaperones with small molecular mass bind to peptide substrates in ATP-dependent cycles in order to shield aggregation prone hydrophobic regions of the target protein (Yon, 2001). On the other hand, large chaperones lock up the unfolded proteins in a cage, giving the peptides an opportunity to refold (Ranford *et al.*, 2000). There is evidence that it is the smaller molecular chaperones that bind the hydrophobic regions of non-native protein before the larger ones are involved. This is because the order, by molecular mass, in which chaperones are added *in vitro,* is essential for the folding process (Kudlicki *et al.*, 1994).

The heat shock response is highly conserved across species of living organisms and because of this there is a growing interest in using the stress induced expression of some molecular chaperones as stress biomarkers (Rajdev and Sharp, 2000). However, the expression profiles of molecular chaperones depend on the nature of stimuli, indicating that genes governing the expression of these proteins are regulated through several pathways (de la Serna *et al.*, 2000, Müller *et al.*, 2000; Rajdev and Sharp, 2000). Consequently, a particular form of physiological stress is able to introduce a unique shift in the balance between chaperones, their co-chaperones and substrates. This results in the establishment of chaperone responses that are tailored to meet the specific physiological demands of the cell under different circumstances (Nollen and Morimoto, 2002).

1.3 Heat shock proteins

1.3.1 Heat shock proteins as molecular chaperones

The term Heat shock protein (Hsp) came into use when it was observed that exposure to heat shock led to production of chromosomal puffs in the salivary glands of *Drosophila* (Ritosa, 1962). Heat shock proteins are ubiquitous and the DNA sequences that make up this family of genes are highly conserved across species. It is interesting to note that events that promote protein misfolding in the cell switch the heat shock regulon on (Parsell and Sauer, 1989). Although heat shock proteins constitute the biggest fraction of molecular chaperones, not all chaperones are heat shock proteins (Hendrick and Hartl, 1993).

1.3.2 Heat shock proteins nomenclature

Heat shock proteins are named according to their molecular sizes ranging from 8 – 150 kDa and major subfamilies are the small heat shock proteins, Hsp40, Hsp60, Hsp70, Hsp90 and Hsp100. Molecular chaperones that are constitutively expressed are generally referred to as heat shock cognate (Hsc) proteins, while those that are stress induced are designated as Hsp proteins. Another system of classifying these proteins is based on their roles (Stirling *et al.*, 2003). This system divides chaperones into holding, folding and unfolding chaperones. Holding chaperones are mostly those that lack ATP-driven conformational changes. Their role is merely to bind, and stabilise non-native proteins for refolding by other chaperones. An example of a holding chaperone is Hsp40 (called DnaJ in prokaryotes), which co-operates with ATP-dependent Hsp70 (called DnaK in prokaryotes) during the folding process (Mayer

et al., 2000a). Small heat shock proteins also fit into this category since they bind and trap denatured proteins onto their surfaces (van Monfort *et al.*, 2001). Folding chaperones are those that are capable of capturing and folding nascent peptides such as Hsp70 (Mayer *et al.*, 2000a), which binds and refolds its peptide substrates. The Hsp100/Clp/AAA (ATPase associated with various cellular activities) family is an example of the unfolding chaperone group that is implicated mostly in protein unfolding and disassembly (Stirling *et al.*, 2003).

1.4 Major groups of heat shock proteins

1.4.1 Hsp100

Members of the Hsp100 group of chaperones are involved in diverse biochemical reactions with the disassembly of quaternary polypeptide complexes (Pak and Wickner, 1997) as their main role. In yeast cells Hsp100 deficiency results in severe susceptibility to heat stress (Sanchez and Lindiquist, 1990). The prokaryotic Hsp100, ClpB (caseinolytic protease B) displays detectable basal and peptide substrate stimulated ATPase activity (Woo *et al.*, 1992). The N-terminal segment of ClpB is essential for peptide substrate binding (Barnett *et al.*, 2005). Like DnaK, ClpB depends on bound nucleotide to regulate its substrate binding kinetics; strangely, it displays higher affinity for peptide substrate in the ATP-bound state and low affinity for the substrate in the ADP bound state (Bösl *et al.*, 2005).

The ability of *Escherichia coli* DnaK (Hsp70 homologue) to disentangle protein aggregates is limited, and therefore requires the intervention of ClpB to assist with reversing aggregation (Goloubinoff *et al.*, 1999). The mechanism of the concerted action between ClpB and the DnaK has been proposed (Goloubinoff *et al.*, 1999). According to this hypothesis, ClpB is the one that interacts directly with aggregated proteins. ClpB undergoes ATP driven conformational changes that enable it to expose the hydrophobic parts of the aggregates, allowing the DnaK to recognise and bind to the complex, consequently reversing aggregation. Another contesting view, the 'crowbar and ratchet' mechanism, proposes that the conformationally induced physical movements of the subdomains of ClpB fragment the protein aggregates in a 'crowbar and ratchet' fashion (Glover and Tkach, 2001). It has also been proposed that ATP induces domain movements causing the axial channel of this protein to move, disintegrating protein aggregates through a 'threading mechanism' (Lum *et al.*, 2004).

1.4.2 Hsp90

This is a highly conserved group of proteins that occurs in all organisms. Initial work on the crystal structure of the N-terminal domain of Hsp90 revealed a dimeric structure anchored on a highly twisted 16-stranded β-sheet (Prodromou *et al.*, 1997). Hsp90 is made up of three subdomains (25 kDa N-terminal ATP-binding domain, 35 kDa middle domain, and a 12 kDa C-terminal domain) all of which are highly conserved (Terasawa *et al.*, 2005). The C-terminal domain is crucial for the dimerisation of Hsp90 (Nemoto *et al.*, 1995). All the three domains of Hsp90 are involved in peptide binding (Scheibel *et al.*, 1999; Sato *et al.*, 2000; Terasawa *et al.*, 2005). It is believed that the transient dimerisation of Hsp90 through the middle and the N-terminal domains is essential for the ATPase activity of this protein (Prodromou *et al.*, 2000; Meyer *et al.*, 2003; Wegele *et al.*, 2003). This has led to speculation that Hsp90 acts as a molecular 'clamp' whose operation is driven by its ATPase activity, which is regulated through the momentary opening and closing events (Prodromou *et al.*, 2000). In turn, the closing and opening cycles are regulated by a cohort of co-chaperones that occur in multicomponent systems (Pearl and Prodromou, 2001; Pratt and Toft, 2003).

Eukaryotic Hsp90 participates in the conformational regulation of signal transduction molecules, such as tyrosine kinases and steroid hormone receptors (Caplan, 1999). In this process, steroid hormone receptors associate with Hsp90 in order for them to adopt conformational competence for hormone binding (Dittmar and Pratt, 1997). Recent findings by Sollars *et al.* (2003) suggest that Hsp90 acts as a 'capacitor' for morphological evolution through epigenetic and genetic mechanisms. The inhibition of the activity of Hsp90 effected changes on the chromatin state that were accompanied with abnormal phenotypic changes in *Drosophila melanogaster* (Sollars *et al.*, 2003). The phenotypic changes persisted in successive generations as heritable traits even after the restoration of Hsp90 activity. Queltsch *et al.* (2002) made similar observations and proposed that Hsp90 serves as a regulatory system at the genotype-environment interface. The 'capacitor' role of Hsp90 is based on its chaperone properties, which allows it to make available functionally competent client proteins at the right time and place during the development process.

1.4.3 Hsp60

Under this group, two proteins are prominent, prokaryotic GroEL/GroES (Viitanen *et al.*, 1990) and eukaryotic t-complex polypeptide-1 (TCP-1 Complex; McCallum *et al.*, 2000). Eukaryotic TCP-1 is a hetero-oligomeric complex, which facilitates folding of different peptides of which tubulin and actin are most distinct (Melki and Cowan, 1994). TCP-1 also closely associates with nascent peptides coming off the ribosome (McCallum *et al.*, 2000). The prokaryotic members of this group of chaperones are involved in the assembly of large multi-protein complexes in which chaperonin 60 (GroEL) forms complexes with chaperonin 10 (GroES) (Viitanen *et al.*, 1990). This results in a double ring structure comprising 14 subunits, which form a large central cavity in which the unfolded protein substrate binds via hydrophobic interactions (Fenton *et al.*, 1994). Each subunit of the GroEL has three domains: an apical domain, to which both substrate and GroES bind; an equatorial domain, which contains a binding site for ATP and the contacts for ring binding; and the intermediate domain which connects the two. The intermediate domain acts as a hinge, effecting conformational changes when ATP is bound (Ranson *et al.*, 1998), causing the substrate-binding surface to alternate between hydrophobic and hydrophilic states. When the surface is hydrophobic, the peptide substrate binds to GroEL, thereby preventing the incorrect association of the substrate with other proteins. As testimony of its importance, the GroEL/GroES chaperone system is indispensable at all temperatures in *E. coli* (Fayet *et al.*, 1989).

When ATP binds to GroEL, the hinge opens up, altering the substrate-binding surface such that it becomes hydrophilic, and the protein substrate is released (Inobe *et al.*, 2001). A mechanism to explain how GroEL encapsulates non-native peptide substrates without allowing it to get into solution has been proposed (Ueno *et al.*, 2004). The binding event occurs in two cycles (Ueno *et al.*, 2004). In the first phase of events, GroEL binds to ATP, peptide substrate, and GroES. In the second phase, the substrate is released into the cavity and only then does folding proceed. Several peptide substrates that interact with GroEL in *E. coli* cytosol have been established and most of them have several domains with α/β subdomains (Houry *et al.*, 1999). GroEL interacts with about 300 newly translated polypeptides, one third of which are structurally unstable, hence are constantly maintained by the chaperonin in their life time (Houry *et al.*, 1999). Thus, it appears that GroEL facilitates the folding of aggregation prone species of proteins as well as those whose recovery to native

state is very slow (Houry *et al.*, 1999). It is not surprising that an environment far from the crowded cellular environment is suitable for refolding such proteins, hence the term "Anfinsen cage" is often used to describe the GroEL/GroES protein refolding complex (Ellis, 1994).

1.4.4 Hsp40

1.4.4.1 Structure of Hsp40 proteins and their classification

The Hsp40 (called DnaJ in prokaryotes) family consist of several unique members defined by the presence of a highly conserved J domain of approximately 70 residues (Laufen *et al.*, 1999). A major role of DnaJ on the functional cycle of DnaK has been found to be the significant stimulation of the ATP hydrolysis step, leading to stabilisation of the DnaK-substrate protein complex (McCarty *et al.*, 1995).

A classification system for the many and varied Hsp40-like proteins is now in place (Figure 1.1; Cheetham and Caplan, 1998). In this classification system all Hsp40-like proteins belong to one of the following three groups: type I Hsp40-like proteins have the same domain structure as the standard *E. coli* DnaJ over their full length containing a J domain, a glycine-phenylalanine (G/F)-rich region, cysteine repeat domain and a C-terminal domain. Type II proteins have similarity to DnaJ over the J domain and the G/F-rich region. Type III proteins are those with only a J domain in common with DnaJ, present anywhere along their structure. Generally, type I and II Hsp40-like proteins are functionally similar; both groups bind to non-native substrate, eventually handing them over to Hsp70 (Walsh *et al.*, 2004). However, type III Hsp40-like proteins are functionally distinct, and do not seem to bind non-native peptides although they present themselves in time and space for the stimulation of their Hsp70 partners (Walsh *et al.*, 2004). An example of type III Hsp40 system is the yeast Hsp40 Swa2p and mammalian axullin, both of which have a J domain and a peptide binding domain that is specialised to cooperate with Hsp70 in uncoating clathrin-coated vesicles (Ungewickell *et al.*, 1995; Gall *et al.*, 2000; Xiao *et al.*, 2006).

A study has shown that both the N-terminal J domain and the adjacent G/F-rich region of DnaJ are required for interaction with DnaK (Karzai and Mcmacken, 1996). However, the J domain on its own has been established as the minimal structural requirement for interaction and stimulation of a partner Hsp70 (Landry, 2003). Although the G/F-rich region of Hsp40

proteins is not essential for the stimulation of Hsp70 (Chamberlain and Burgoyne, 1997), it has been implicated in the modulation of DnaK's interaction with substrate (Wall *et al.*, 1995). Domain swapping experiments have revealed that the G/F-rich region is crucial for the complete *in vivo* function of DnaJ, and in addition, this motif confers functional specificity to DnaJ (Cajo *et al.*, 2006).

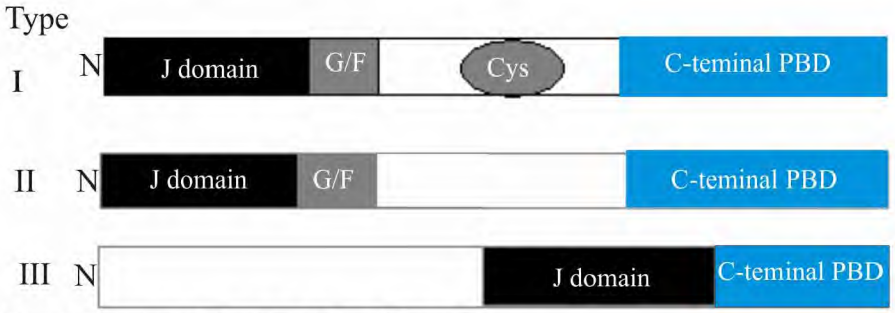

Figure 1.1 Structure and classification of Hsp40 proteins
Linear representation of Hsp40 showing the different subdomains that characterise each type of Hsp40 protein. The different subdomains illustrated here are as follows: the J domain, glycine/phenylalanine-rich region (G/F), cysteine-rich region (Cys) and the C-terminal peptide binding domain (C-terminal PBD). The N-terminus is shown as 'N' (adapted from Cheetham and Caplan, 1998).

A highly conserved segment, the DIF motif, has been identified within the G/F region of DnaJ (Wall *et al.*, 1995). The G/F region of *E. coli* DnaJ has three such motifs and interestingly, single amino acid mutations on this motif produced functional deficiencies equivalent to the G/F-rich deletion mutant (Cajo *et al.*, 2006). It has been hypothesised that the DIF motif of the DnaJ G/F-rich region, though not directly involved in the stimulation of the ATPase activity of DnaK, plays a role at some point leading to ATP hydrolysis by DnaK (Cajo *et al.*, 2006). In some human Hsp40s the G/F-rich region serves as a flexible spacer between the J domains and C-termini of these proteins (Borges *et al.*, 2005). The G/F-rich region confers a distinct quaternary structure to Hsp40 proteins, consequently providing them with unique substrate binding capabilities (Borges *et al.*, 2005).

The cysteine-rich subdomain of DnaJ forms a zinc finger motif which facilitates the interaction of DnaJ with Zn(II) metal ions; qualifying DnaJ as a metalloprotein (Banecki *et al.*, 1996). Two Zn(II) metal ions bind to a monomer of DnaJ (Banecki *et al.*, 1996). The deletion of the cysteine-rich region of DnaJ did not affect DnaJ's affinity for DnaK, however, it reduced the stability of this interaction (Banecki *et al.*, 1996). A DnaJ whose cysteine-rich region was removed had reduced affinity for some of its peptide substrates (Banecki *et al.*, 1996) and it is thought in Ydj1 (a yeast Hsp40 homologue), the cysteine-rich region closely

co-operates with the C-terminal subdomain and both domains assist Hsp70 in protein folding (Lu and Cyr, 1998). The C-terminal subdomain is responsible for interaction with substrate and is the major determinant of substrate specificity for type I and II Hsp40 proteins (Green *et al.*, 1998). Indeed, removal of the C-terminal peptide binding domain from Hsp40 compromises its function (Johnson and Craig, 2001). It is this segment of Hsp40 that is responsible for self-association, an essential feature for Hsp40 chaperone activity (Shi *et al.*, 2005).

1.4.4.2 Hsp40 as a co-chaperone of Hsp70

The Hsp70 protein interacts with peptide substrate in ATP expending cycles. Hsp40 acts as a co-chaperone of Hsp70, and is capable of stimulating ATP hydrolysis (McCarty *et al.*, 1995), interacting with the ATPase domain of Hsp70 through a channel that is located in the ATPase segment of the protein (Gässler *et al.*, 1998). There is evidence that Hsp40 also interacts with the peptide binding domain of Hsp70 (Suh *et al.*, 1998; Davies *et al.*, 1999). Peptide binding through the C-terminal peptide binding domain also raises the ATPase activity of Hsp70 (Slepenkov and Witt, 2003). Thus, maximum activation of Hsp70 is attained by two closely coupled events - Hsp40 stimulation and substrate binding. The advantage of this mechanism for stimulation of Hsp70 being that it ensures that Hsp70 is activated only by Hsp40-supplied substrates thereby avoiding unnecessary energy consuming cycles (Kelley, 1999). Although Hsp40 acts as a co-chaperone for Hsp70 (McCarty *et al.*, 1995), evidence suggests that DnaJ and other members of the Hsp40 family are chaperones in their own right, binding some unfolded peptides and reversing their aggregation *in vitro* (Schröder *et al.*, 1993).

1.4.4.3 Hsp40-Hsp70-substrate complex formation

The presence of large hydrophobic residues in client peptides is the basis for their recognition by both Hsp70 and Hsp40 chaperones (Takenaka *et al.*, 1995; Li and Sha, 2004). However, there is a slight variation between DnaK and DnaJ's typical peptide substrates (Rüdiger *et al.*, 2001). DnaJ has a longer hydrophobic core on its substrate binding motif than DnaK and DnaJ favours substrates rich in aromatic residues as opposed to DnaK's leucine-rich substrates (Rüdiger *et al.*, 1997; Rüdiger *et al.*, 2001). Furthermore, DnaK is sensitive to the orientation of its substrates in space, and in contrast DnaJ binds both D- and L- oriented peptides (Rüdiger *et al.*, 2001). Despite the small divergences in the motifs of their

substrates, DnaK and DnaJ can bind the same substrates (Rüdiger *et al.*, 2001). It has been shown that DnaK and DnaJ can interact with the same substrate through different sites (Kim *et al.*, 2002). The question that arises is which of the two binds substrate first? One view advocates that Hsp40 binds non-native proteins first and brings them within reach of the Hsp70 protein (Hartl, 1996; Rüdiger *et al.*, 2001). The fact that DnaJ recognises both D- and L-oriented substrates, binding to them loosely compared to DnaK whose grip on its substrates is firm, has been presented as evidence that DnaJ merely acts as a substrate 'scanner' for DnaK (Rüdiger *et al.*, 2001).

Another proposal is that both DnaK and DnaJ associate with a substrate at the same time through different sites (Rüdiger *et al.*, 2001; Han and Christen, 2003). According to this proposal, both ATP-DnaK and DnaJ bind to the same peptide substrate simultaneously forming $(\text{ATP-DnaK})_m$.substrate-DnaJ_n complex (Han and Christen, 2003). It is this complex that then facilitates maximum interaction between DnaJ and DnaK through a cis-transposition that allows the J domain of DnaJ to bind DnaK (Han and Christen, 2003). Additional support for the second proposal emanates from the fact that DnaK's basal affinity for peptide substrate is an important factor in the chaperone role of DnaK (Mayer *et al.*, 2000b). Han and Christen (2003), further postulate that a single DnaJ molecule interacts sequentially with several DnaK molecules; this is logical since cells have less molar DnaJ ratio than DnaK (Neidhardt *et al.*, 1984). The fact that the only the J domain is conserved across Hsp40-like proteins (Laufen *et al.*, 1999) whilst great variation occurs in their substrate binding domain does not support their role as Hsp70 substrate scavengers (Rüdiger *et al.*, 2001). However, only type I and type II Hsp40s can present substrates to Hsp70, whilst type III have not been shown to play this role (Walsh *et al.*, 2004). This is because only type I and type II Hsp40s have both the ability to modulate Hsp70 chaperone activity as well as being able to bind substrates (Walsh *et al.*, 2004).

Most of the studies on Hsp40-Hsp70 interaction were based on work done using the *E. coli* system. However, there is work that has been conducted on the eukaryotic system that sheds light on how this system operates. Yeast Hsp40s, like their bacterial counterparts (Rüdiger *et al.*, 2001; Li and Sha, 2004), possess hydrophobic patches on their surfaces, enabling them to bind peptides rich in bulky hydrophobic residues (Lee *et al.*, 2002). In both bacterial and

yeast systems, a bipartite signalling mechanism in which their respective Hsp40s interact with their Hsp70 counterparts through both the ATPase and C-terminal domains has been observed (Karzai and McMacken, 1996; Aron *et al.*, 2005). The contact point between *Saccharomyces cerevisiae* DnaJ homologue, Sis1 and the C-terminal domain of yeast Hsp70, Ssa1 has been determined and the interaction is based on ionic forces (Qian *et al.*, 2002). The bipartite signalling system is believed to facilitate peptide substrate transfer between Hsp40 and Hsp70 (Aron *et al.*, 2005). A mechanism to explain how this happens, 'the anchoring and docking mechanism' proposes that the C-terminus of the Hsp70 protein forms an anchoring motif which docks Hsp70 with Hsp40, thus facilitating substrate transfer (Qian *et al.*, 2002). This association ends when ATP gets hydrolysed, leading to the closure of the Hsp70 protein's lid and the dissociation of the Hsp70 protein from Hsp40 (Qian *et al.*, 2002). Therefore, in spite of their structural diversity across different species, Hsp40s display strikingly conserved mechanisms of action as they cooperate with their Hsp70 counterparts during peptide folding (Fan *et al.*, 2003). However, most of the current understanding on the mechanistic nature of Hsp40/Hsp70 partnership is limited as it is based mostly on studies involving type I and type II Hsp40s.

Generally, the pool of Hsp40 species in a cell tend to outnumber that of Hsp70s, particularly in eukaryotes were several Hsp40 homologues occur in a single cell (Ohtsuka and Hata, 2000). It has been established that one Hsp70 protein can interact with more than one Hsp40 co-chaperone, and thus the large pool of Hsp40s in eukaryotic cells ensures a diversity of functional partnerships between the few Hsp70s and the several Hsp40 proteins (Cyr, 1995; Walsh *et al.*, 2004). Furthermore, since eukaryotic cells possess several subcellular organelles, the requirement for organelle specific Hsp40/Hsp70 functional partners such as those in the endoplasmic reticulum (ER) and mitochondria of eukaryotic cells (Schlenstedt *et al.*, 1995; Voisine *et al.*, 2001; Shen and Hendershot, 2005) ensures the availability of a specialised chaperone system that can meet the protein folding demands of these organelles.

1.4.5 Small heat shock proteins

Small heat shock proteins occur in all organisms and have molecular weight that ranges between 16 - 30 kDa. Most small heat hock proteins form oligomeric complexes (de Jong *et al.*, 1998). One of the most extensively studied of this group of chaperones is α-crystallin

which has the ability to interact with unfolded protein thereby preventing further aggregation (Horwitz, 1992). Small heat shock proteins contain the α-crystallin domain as a common feature, which is located on the C-terminal end in most cases (Gusev *et al.*, 2002), and perhaps for this reason the C-terminus is more crucial for function than the N-terminus in these chaperones (Fernando and Heikkila, 2000). Steady state mRNA data on the expression of some small heat shock proteins suggested that they are predominantly produced in skeletal, heart, placenta, spleen and smooth muscles of humans (Kappe *et al.*, 2001). Because of the high stress levels associated with muscle tissue, it is likely that small heat shock proteins play an important role in the stress response of these tissues (Sugiyama *et al.*, 2000).

1.4.6 Hsp70

1.4.6.1 The role of Hsp70

Hsp70 is a group of highly ubiquitous proteins, localised in the *E. coli* cytosol and some compartments of eukaryotic cells such as endoplasmic reticulum lumen, mitochondrial matrix, and the cytosol (Table 1.1; Johnson and Craig, 1997). The compartmentalisation of Hsp70 protein homologues in eukaryotic cells ensures specialised cellular roles for these proteins (Craig *et al.*, 1994).

Immuno-precipitation experiments have demonstrated Hsp70's ability to associate with a wide spectrum of polypeptides larger than 20 kDa (Thulasiraman *et al.*, 1999). In eukaryotes, Hsp70s are implicated in a wide range of activities within the cell such as folding of nascent polypeptides (Eggers *et al.*, 1997), disaggregation of protein units (Song *et al.*, 2005), protein translocation (Gambill *et al.*, 1993), protein degradation (Bercovich *et al.*, 1997), signal transduction (Asea *et al.*, 2002) and DNA replication (Song *et al.*, 2005). In *E. coli*, DnaK occurs abundantly under both stress and non-stress conditions constituting about one percent of total protein during the exponential growth phase (VanBogelen *et al.*, 1997). Although DnaK is associated with newly synthesised nascent polypeptides (Teter *et al.*, 1999), *E. coli* cells lacking DnaK can survive under normal conditions (Paek and Walker, 1987). However, *E. coli* cells devoid of DnaK are heat sensitive (Paek and Walker, 1987; Kusukawa and Yura, 1988) and they display other wide ranging physiological defects amongst them are temperature sensitivity, defects in chromosomal segregation and poor plasmid maintenance (Bukau and Walker, 1989a). The fact that nascent chains in eukaryotes tend to remain bound

to their Hsp70 chaperone much longer than in prokaryotes suggests a more pronounced role for these chaperones in eukaryotes than in prokaryotes (Feldman and Frydman, 2000).

Table 1.1 Hsp70 homologues from bacteria, yeast and mammals

Hsp70	Location	Function	References
		Bacteria	
DnaK	Cytosol	-Refolding nascent peptides	Hartl and Hayer-Hartl (2002)
Hsc62 (HscC)	Cytosol	-Regulates transcription	Arifuzzaman *et al.* (2002)
Hsc66 (HscA)	Cytosol	-Reversing protein aggregation, involved in iron cluster protein assembly	Silberg *et al.* (1998); Hoff *et al.* (2000)
		Yeast	
Ssa1 – Ssa4	Cytosol	-Folding and translocation of peptides	Shulga *et al.* (1996); Kim *et al.* (1998)
Ssb1-2, Ssz1	Cytosol	-translation of proteins	Nelson *et al.* (1992); Hundley *et al.* (2002)
Ssc1-2, Ssq1	Mitochondria	-protein biogenesis, folding and translocation	Gambill *et al.* (1993); Knight *et al.* (1998); Dutkiewicz *et al.* (2006)
Lhs1p	ER	-Protein folding and translocation into ER	Craven *et al.* (1996)
Kar2p, (Ssi1p, Cer1p)	ER	-Protein folding and translocation into ER	Baxter *et al.* (1996); Hamilton and Flynn (1996)
		Mammals	
Hsp72, Hsc73	Cytosol/Nucleus	-Folding and translocation of polypeptides	Ellis *et al.* (2000); Goldfarb *et al.* (2006)
mtHsp70	Mitochondria	-Protein translocation	Schneider *et al.* (2002)
BiP (Grp78)	ER	-Folding and translocation of peptides	Hendershot *et al.* (1996); Lièvremont *et al.* (1997)
Grp170	ER	-Protein translocation	Dierks *et al.* (1996)

Some of the roles of DnaK, include: folding of newly synthesised proteins coming off the ribosomes and refolding of denatured proteins (Skowyra *et al.*, 1990; Hartl and Hayer-Hartl, 2002); facilitating DNA supercoiling (Ogata *et al.*, 1996); ribosomal biogenesis (El Hage *et al.*, 2001); chromosomal segregation (Bukau and Walker, 1989a); and signal transduction (Kelley and Georgopoulos, 1997). DnaK protects cells not only through prevention of aggregation but by refolding non-native proteins as well (Skowyra *et al* 1990). DnaK is essential for protein complex assembly, for example, DnaK is involved in *E. coli* β-Galactosidase α-complementation (Ferreira and Alix, 2002), a process in which two subunits

of this protein noncovalently come together resulting in a functional enzyme. DnaK is essential for this process through its ability to catalyse the intramolecular organisation of protein subunits (Lund, 2001). In *E. coli*, DnaK together with DnaJ and DnaK's NEF, GrpE, modulate the heat shock response (Straus *et al.*, 1990). The modulation of the heat shock response by these chaperones is based on their ability to regulate the synthesis and stability of sigma 32, a molecule that is important for initiation of the heat shock response (Straus *et al.*, 1990). Indeed, even the smallest changes in the levels of DnaK and DnaJ (for example, due to the titration of these chaperones by misfolded proteins at high temperatures), can register detectable feedback on the heat shock response (Tomoyasu *et al.*, 1998).

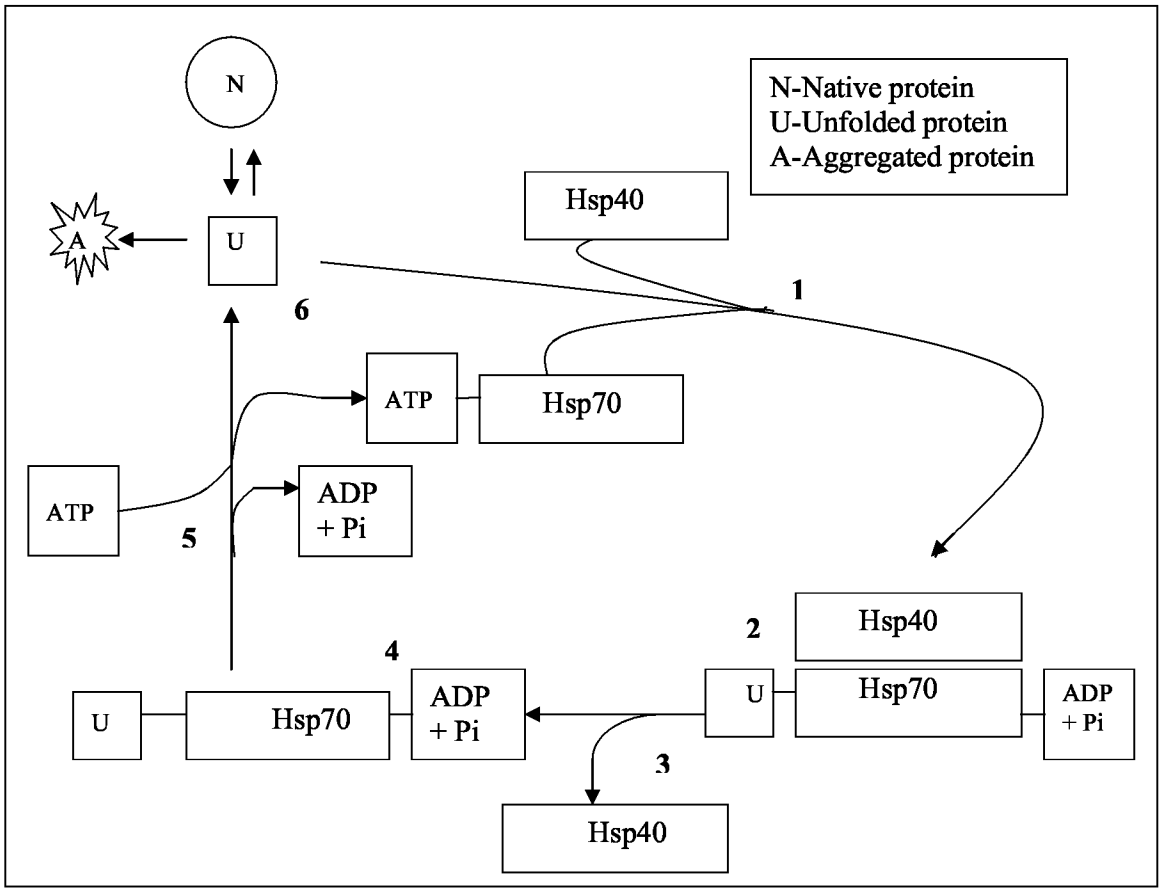

Figure 1.2 Hsp70/DnaK functional cycle
(1) Unfolded peptide interacts with Hsp40 and Hsp70 through its exposed hydrophobic patches, (2) Hsp40 stimulates hydrolysis of ATP by Hsp70, (3) Hsp40 leaves the complex, (4) The ADP bound Hsp70 has high affinity for substrate facilitating substrate refolding, (5) NEF facilitates ADP replacement by ATP on Hsp70, (6) the now natively folded protein is released, whilst protein misfolded beyond refolding is sent for degradation (adapted from Fan *et al.*, 2003)

In its functional cycle, Hsp70 binds to peptide substrate, allowing it to refold, followed by release of the substrate through ATP-expending cycles (Figure 1.2; Szabo *et al.*, 1994). The

nucleotide to which Hsp70 is bound regulates substrate affinity by Hsp70. In the ADP bound state, Hsp70 has high affinity for the peptide substrate whilst its affinity for the substrate is reduced in the ATP bound state (Suh *et al.*, 1999). ATP binding induces a conformational change that transcends to the peptide binding domain resulting in Hsp70 having low affinity for substrate leading to release of substrate (Liberek *et al.*, 1991). In order for the cycle to proceed, nucleotide exchange has to occur. GrpE facilitates nucleotide exchange in *E. coli* (Harrison *et al.*, 1997).

1.4.6.2 Structural and functional features of Hsp70

Most Hsp70 proteins have a molecular mass of 70 kDa and consist of two distinct domains; the 45 kDa N-terminal domain that binds ATP, and 25-kDa peptide binding domain (Figure 1.3A; Flahety *et al.*, 1990; Wang *et al.*, 1993). *E. coli* DnaK has an ATPase domain consisting of residues 1-385, followed by a highly conserved linker region (residues 388-389) connecting the ATPase domain to the peptide binding domain. The peptide binding domain of DnaK is further subdivided into the β-sandwich (residues 389-508) and the lid segment (residues 509-638), which is further subdivided into α-helical domains A-E, and a C-terminal segment of about 30 residues which is structurally unresolved (Figure 1.3).

1.4.6.2.1 The ATPase domain

An ATPase domain of common origin occurs in proteins with a diverse functional base, such as actin, hexokinase and Hsp70 (Bork *et al.*, 1992a). Sequence alignment and three dimensional models for the ATPase domains of these proteins have revealed the existence of groups of highly conserved amino acid residues that are organised into five motifs that are involved in ATP binding (Figure 1.3; Bork *et al.*, 1992a). The ATPase domain of Hsp70 has two domains of a similar fold whose convergence point creates a cleft that accommodates a nucleotide (Figure 1.3B; Flaherty *et al.*, 1990; Bork *et al.*, 1992a). The two domains are in turn subdivided into four subdomains (subdomain IA, IIA, 1B and IIB) (Figure 1.3B; Bork *et al.*, 1992a). Both DnaJ and GrpE have binding sites located in the ATPase domain of DnaK (Buchberger *et al.*, 1994; Gässler *et al.*, 1998). Besides the regulation of the peptide binding kinetics of Hsp70 proteins, a study on the constitutive form, Hsc70, and *E. coli* DnaK showed that ATP binding favours the monomeric form of the protein (Palleros *et al.*, 1993;

Gao *et al.*, 1996). Otherwise Hsp70 is stored in its oligomeric state which does not bind peptide substrate (Gao *et al.*, 1996).

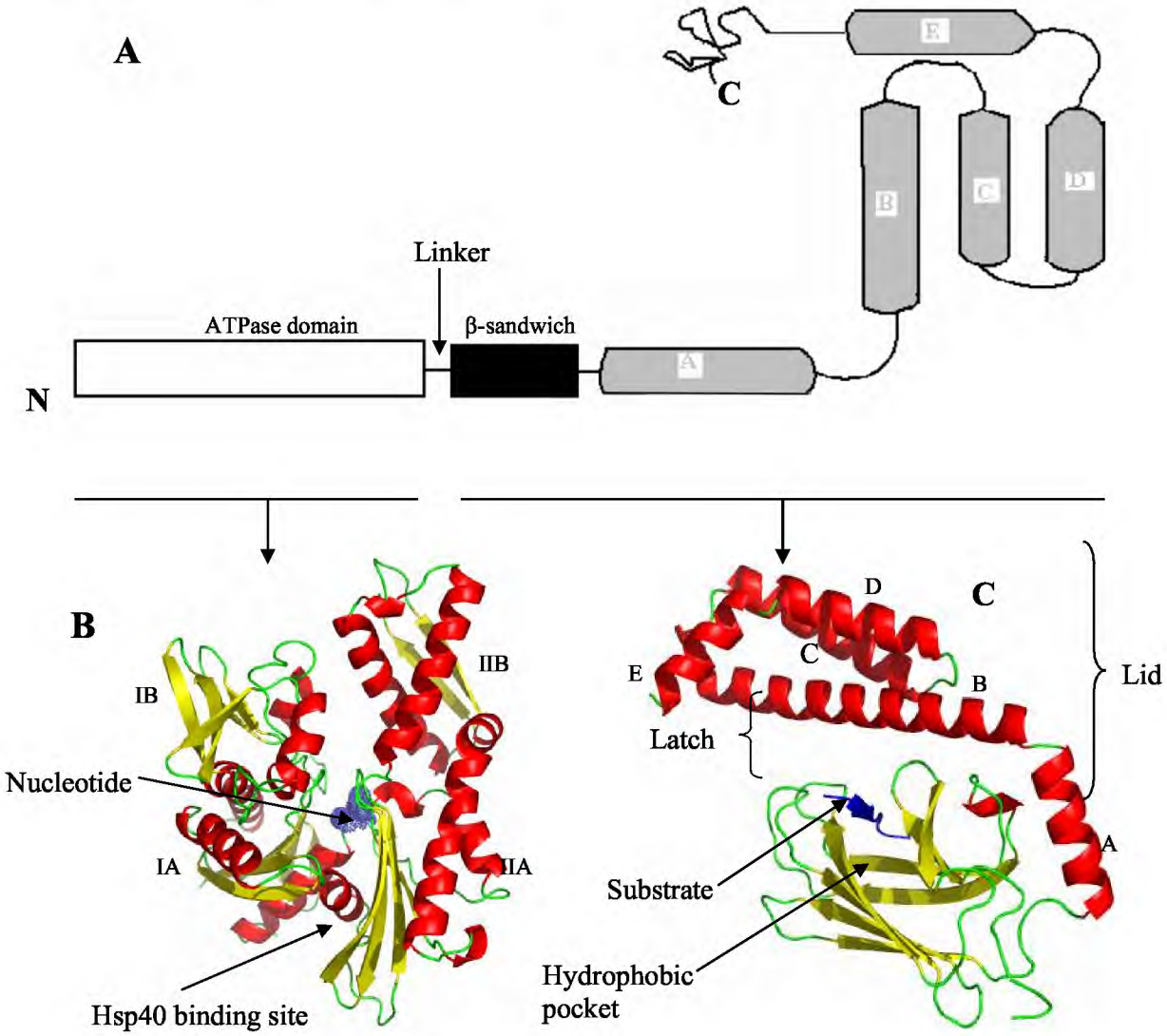

Figure 1.3 Domain structure of Hsp70
(**A**) Schematic representation of the domain organization of DnaK. The figure shows the N-terminal ATPase domain linked to the peptide binding domain through a highly conserved linker region. The C-terminal peptide binding domain is further subdivided into the β-sandwich and the lid segments which is constituted of helical subdomains A-E, and a structurally unresolved C-terminus of about 30 residues. (**B**) Ribbon representation of the ATPase domain of Hsc70 (3HSC.pdb; Flaherty *et al.*, 1990). The N-terminal ATPase domain is made up of two adjacent domains, each subdivided into two subdomains (subdomain IA and IB make up the left lobe whilst subdomains IIA and IIB make up the right lobe; Bork *et al.*, 1992a). Shown in blue colour is the nucleotide, and the Hsp40 binding cleft is located at the bottom of the structure (Gässler *et al.*, 1998). (**C**) Ribbon representation of the peptide binding domain of *E. coli* DnaK bound to peptide substrate. The peptide binding domain of DnaK interacts with peptide substrate (blue) through the hydrophobic sandwich formed by the β- sheets. The α-helical lid then locks the peptide substrate in the hydrophobic pocket. Salt bridges and hydrogen bonds between residues of helix B of the lid and the outer loops of the peptide binding domain form the latch. The N- and C-termini are shown as 'N' and 'C', respectively (1DKX.pdb; Zhu *et al.*, 1996). The figure was visualised using PyMol (DeLano, 2002).

Although it is widely believed that the specificity of interaction between the peptide substrate and the Hsp70 partner is largely determined by the peptide binding domain, there are exceptions to this. For example, a murine Hsp70 ATPase domain, and not its peptide binding domain, harbours the minimum structural features to enable it to recognise a sulphogalactolipid (Mamelak and Lingwood, 2001). Another Hsp70, Ssz1 from yeast does not need its peptide binding domain for function, suggesting that it binds to its substrate in a fashion different from that which is known for most Hsp70s (Hundley *et al.*, 2002).

1.4.6.2.2 The peptide binding domain of Hsp70

The peptide binding domain of Hsp70 consists of β-sheets followed by a long α-helix, commonly referred to as the lid (Figure 1.3; Zhu *et al.*, 1996). The peptide binding domain of DnaK is not only important in the substrate binding process but is also capable of carrying out minimal refolding of substrates *in vitro* in the absence of the ATPase domain suggesting that polypeptide binding is the least event necessary for refolding to occur (Tanaka *et al.*, 2002). Consequently, the overproduction of the C-terminal domain (G384 to K638) in *E. coli* led to cell death, which is thought to have been linked to its ability to excessively sequester proteins from the cell (Burkholder *et al.*, 1996).

The β-subdomain and residues that have physical contacts with the substrate are conserved across species and greater variation is observed outside the binding cavity (Zhu *et al.*, 1996). DnaK has important structural features important for its interaction with substrate: a deep pocket that accommodates a single hydrophobic residue and an arch formed by residues M404 and A429 (Mayer *et al.*, 2000b). The introduction of steric hindrance in the arch through a V436F substitution abrogated the *in vivo* function of DnaK (Mayer *et al.*, 2000b). The substrate binding cavity of DnaK constantly opens and closes both in the ATP and ADP bound states of the protein (Mayer *et al.*, 2000b). However, in the ATP-bound state, the substrate binding cavity attains the open conformation more frequently than the closed state; allowing the substrate to escape (Mayer *et al.*, 2000b).

The precise role of the lid segment (Figure 1.3C) in Hsp70 has been shrouded in mystery. However, lately, there has been growing interest to elucidate its role. A lidless form of DnaK (residues 1-507) managed to support lambda growth *in vivo*, albeit less effectively than the

wild type (Pellecchia *et al.*, 2000). Interestingly a closely related variant, DnaK (residues 2-538) could not refold a peptide substrate *in vitro* (Mayer *et al.*, 2000b). In addition, lidless variants of Hsp70 conformationally respond to the presence of ATP in the same way as the wild type (Mayer *et al.*, 2000a). Furthermore, the removal of the lid does not result in DnaK taking the open conformation (Mayer *et al.*, 2000b).

Another study that has been of interest is the elucidation of the role of the lid relative to events in the β-sandwich of Hsp70. Recently, some key residues in the α-helical subdomain B of the lid of DnaK have been implicated in forming ionic contacts with other residues in the β-sheet and in α-helical subdomains C and D (Figure 1.4; Fernandez-Sáiz *et al.*, 2006). Ionic contacts between residues D526 and R445 in the N-terminal region of the α-helical subdomain B and β-sheet subdomain were shown to be important for stabilising the lid, as well as facilitating the interaction between the β-sheet subdomain and the lid of DnaK (Figure 1.4; Fernandez-Sáiz *et al.*, 2006). This observation concurs with another study that noted that the lid plays a role in stabilising the β-sandwich (Moro *et al.*, 2004).

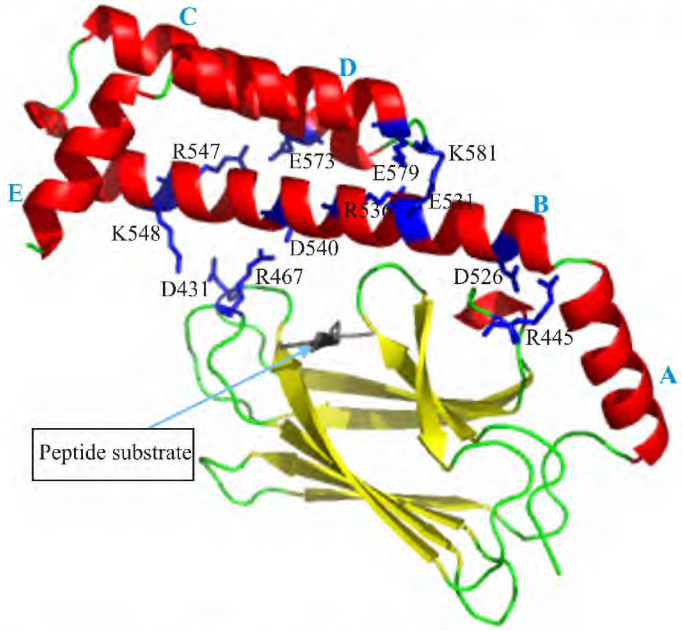

Figure 1.4 Ionic contacts responsible for maintaining DnaK lid stability
Residues that were shown to be important for ionic contacts are shown in blue colour. Some residues in the α-helical subdomain B have partners for ionic interaction is the β-sheet subdomain and others in the α-helical subdomains C and D (Fernandez-Sáiz *et al.*, 2006). The substrate is shown in grey colour and the α-helical subdomains are represented by blue letters, starting from the N-terminal subdomain A to the C-terminal subdomain E (1DKX.pdb; Zhu *et al.*, 1996). The figure was visualised using PyMol (DeLano, 2002).

It has previously been suggested that removal of the lid does not compromise the integrity of the arch of DnaK (Stevens *et al.*, 2003). However, Fernandez-Sáiz *et al.* (2006) observed that residues constituting the latch (D540, K548, D431 and R467; Figure 1.4) controlled access to the hydrophobic binding pocket. The disruption of ionic interactions in this region of DnaK resulted in a protein whose ability to refold substrate was compromised (Fernandez-Sáiz *et al.*, 2006). Not surprisingly, a study by Mayer *et al.* (2000b) showed that truncation of the lid through the mid point of helix B of the peptide binding reduced DnaK's affinity for substrates. In addition, the lid has been implicated in the regulation of the kinetics of substrate recognition and selection (Wu and Wang, 1999; Ohno *et al.*, 2004), consequently influencing the lifespan of the Hsp70/peptide complex (Buczynski *et al.*, 2001; Slepenkov *et al.*, 2003). Therefore evidence for the role of the lid in substrate binding continues to grow, and it seems that each subdomain of the lid contributes uniquely to the integrity of the peptide binding complex (Slepenkov *et al.*, 2003).

1.4.6.2.3 Hsp70-peptide substrate interaction

DnaK binds peptide substrate in an extended conformation (Landry *et al.*, 1992) and the substrate gets accommodated in a cavity that is formed by the strands of the β-sandwich in the peptide binding domain (Zhu *et al.*, 1996). Peptide binding occurs in an induced fit fashion (Mayer *et al.*, 2000a). The binding forces that are responsible for holding the peptide and its Hsp70 partner together are hydrogen bonds, Van der Waals interactions, hydrophobic interactions (Zhu *et al.*, 1996; Rüdiger *et al.*, 1997).

Hsp70 has a high affinity for peptides that are eight residues long, possessing an interior hydrophobic core, which is surrounded by basic residues (Jordan and McMacken, 1995; Gragerov *et al.*, 1994). DnaK motifs occur at an average rate of every 36 residues along a protein's length, leading to speculation that this high frequency of binding sites accounts for DnaK's promiscuity in substrate recognition (Rüdiger *et al.*, 1997). The occurrence of many Hsp70 binding sites along polypeptides could explain why Hsp70 has the ability to recognise and bind short and long peptides with equal efficiency *in vitro* (Flynn, *et al.*, 1989). In fact, Hsp70 recognises so broad a substrate range that it has been suggested that it binds substrates indiscriminately as long as its partner Hsp40 is in the vicinity (Misselwitz *et al.*, 1998; Matlack *et al.*, 1999). However, this claim has been disputed since DnaK has basal affinity

for its substrates whose magnitude should meet a threshold value for its chaperone cycle to proceed (Mayer *et al.*, 2000b).

A mass spectrometric study was used to analyse features of peptides that aggregate in the absence of functional DnaK in *E. coli* (Mogk *et al.*, 1999). The molecular mass profile of the proteins indicated that high molecular weight proteins (>70 kDa) were more represented than smaller proteins (<30 kDa). Therefore, large proteins are potentially the most vulnerable targets of misfolding and consequently constitute the bulk of DnaK substrates in the cell. It has since been established that GroEL and DnaK recognise and refold different substrates (Ewalt *et al.*, 1997). The majority of the *E. coli* thermolabile proteins carry the marker feature for DnaK binding (Rüdiger *et al.*, 1997) as compared to their thermoresistant counterparts (Mogk *et al.*, 1999). On the other hand, GroEL mainly associates with substrates that have several α/β domains (Houry *et al.*, 1999) and facilitates organisation of these subdomains during the assembly of polypeptides (Feldman and Frydman, 2000). An Hsp70 resident in the endoplasmic reticulum, the immunoglobulin heavy chain binding protein (BiP) recognises and binds substrates not merely due to the presence of a BiP binding site on the target peptide, but instead it prefers peptides that fold slowly compared to those that can attain their native state faster (Hellman *et al.*, 1999). Therefore, this endoplasmic reticulum-based Hsp70 is sensitive to the economic demands of the cell during physiological stress.

1.4.6.2.4 The interaction of Hsp70 with nucleotide exchange factors

The contact point through which a NEF interacts with Hsp70 is located in the ATPase domain of Hsp70 (Brehmer *et al.*, 2001). Bag-1 serves as the NEF for mammalian cytosolic Hsc70 and Hsp70 (Höhfeld and Jentsh, 1997; Brehmer *et al.*, 2001). Another mammalian Hsp70 NEF, Hsp70-binding protein (HspBP1), has been identified (Kabani *et al.*, 2002). Shomura *et al.* (2005) showed that HspBP1 acts as more than just a NEF of Hsp70 since it is also involved in the allosteric interaction of the Hsp70. A cofactor of Hsp70, GrpE, has been identified as responsible for facilitating nucleotide exchange in prokaryotes (Harrison *et al.*, 1997). In *E. coli*, DnaK, DnaJ and GrpE occur in the ratio of 10:1:3, respectively (Slepenkov and Witt, 2002). Of the three, it is GrpE, which is essential at all temperatures of growth in bacteria (Ang and Georgopoulos, 1989). At high temperatures, GrpE increases DnaK's chaperone activity by down-regulating its ADP/ATP exchange activity (Grimshaw *et al.*,

2003). GrpE also influences the chaperone activity of Hsp70 protein by regulating its association and dissociation with the peptide substrate independent of ATP (Brehmer *et al.*, 2004). Thus, under low ATP conditions in the cell, GrpE is thought to further slow down substrate release to compensate for the failing ATP driven chaperone activity of DnaK (Brehmer *et al.*, 2004).

Although structurally unrelated to GrpE, Bag-1 can be considered the functional equivalent of GrpE (Hörfeld and Jentsch, 1997). Similarly, the structures of GrpE-DnaK and Bag-1-Hsc70 associations displayed an identical conformational change in the respective ATPase domains (Harrison *et al.*, 1997; Soundermann *et al.*, 2001). On the other hand, the mechanism by which nucleotide exchange occurs on eukaryotic Hsp70 through the action of HspBP1 differs from the GrpE/Bag-1 driven one (Shomura *et al.*, 2005). Furthermore, there is evidence that Hsp70s differ with respect to their intrinsic rate of ADP release, justifying the need for them to have specialised nucleotide exchange systems (Russel *et al.*, 1998; Silberg and Vickery, 2000). Variations within structural features of the NEF binding site of Hsp70s are responsible for providing unique Hsp70-nucleotide exchanger partnerships (Brehmer *et al.*, 2001). For this reason, Brehmer *et al.* (2004) subdivided nucleotide exchange systems in Hsp70s into three prototypes - DnaK proteins, HscA (Hsc66) and Hsc70 proteins as determined by their distinct interaction with GrpE and Bag-1. As a reflection of the divergent nature of NEFs, lumenal Hsp70-1 (Lhs1), an endoplasmic reticulum based Hsp70 interacts with BiP in the endoplasmic reticulum facilitating nucleotide exchange and in return BiP activates the ATPase activity of Lhs1 (Steel *et al.*, 2004).

1.4.6.2.5 Hsp70 interdomain interaction

During its functional cycle Hsp70 undergoes conformational changes that transcend across both the ATPase and peptide binding domains, and this process is important in regulating its chaperone role (Montgomery *et al.*, 1999). A study involving a mammalian Hsc70 revealed that the N-terminal ATPase domain on its own has an ATPase activity several orders of magnitudes higher than the full-length protein without bound peptide substrate (O'Brien and McKay, 1993). It has since been discovered that the interaction between the ATPase and peptide binding domains of Hsp70 influences both the ATPase activity and affinity for the peptide substrate by the protein (Moro *et al.*, 2003; Slepenkov and Witt, 2003).

Since interdomain communication is important for Hsp70 function, it is not surprising that some mutations in the highly conserved linker region of DnaK affect the chaperone role of this protein (Han and Christen, 2001). The conserved linker residues are also implicated in the interaction of DnaJ with DnaK (Laufen *et al.*, 1999; Han and Christen, 2001). The crystallisation of the partial full length of Hsc70 (Figure 1.5) has been successfully carried out and more structural insights involving Hsp70 interdomain communication have since been gained (Jiang *et al.*, 2005). Findings from this work suggest that the interdomain interface is constituted of regions from both the ATPase and peptide binding domains. The Hsc70 crystal structure shows helix A of the lid segment as the biggest constituent of the Hsc70 interdomain interface, and this structure is situated very close to the Hsp40 binding site in the ATPase domain (Jiang *et al.*, 2005). Assuming this structure resembles the three dimensional structure of DnaK, this would explain why DnaK experiences chemical shifts originating in the ATPase domain and extending into the peptide binding region (Buchberger *et al.*, 1995). The proximity of the residue R171 (in the ATPase domain) of bovine Hsc70 to the C-terminal helix A subdomain of the lid (Figure 1.5) confirms the possible involvement of the lid both in the allosteric regulation and the Hsp40 driven Hsc70 conformational changes (Jiang *et al.*, 2005). Residue R167 of DnaK is the equivalence of R171 of the bovine Hsc70 and the role of R167 is essential for the modulation of DnaK by DnaJ (Suh *et al.*, 1998). Furthermore, structural studies suggest that when Hsp40 binds Hsc70, the positively charged J domain of Hsp40 is within the influence of the negatively charged regions of the Hsc70 peptide binding domain, allowing the Hsp40 J domain to physically interact with Hsc70 through its C-terminal domain (Jiang *et al.*, 2005).

The linker moves from an 'exposed' to 'buried' position when ATP binds to Hsp70 (Buchberger *et al.*, 1995; Jiang *et al.*, 2005). Therefore it could be assumed that when ATP is bound in the ATPase domain, the linker responds by making a movement, which transcends into the peptide binding domain (Jiang *et al.*, 2005). In addition, some key residues in the peptide binding domain have been implicated in regulating events in the ATPase domain including the effect of co-chaperones acting through this subdomain (Montgomery *et al.*, 1999; Mayer *et al.*, 2000a). However, the lid is dispensable for interdomain communication in DnaK (Pellecchia *et al.*, 2000). In contrast, another study found that ATP binding in the N-terminal ATPase domain leads to rotation of the lid through a hinge region (amino acids 536-538) that is situated in the middle of helix B (Zhu *et al.*, 1996). Therefore the role of the lid

in interdomain communication remains controversial. However, given the fact that the lid is important for peptide binding (Slepenkov *et al.*, 2003; Fernandez-Sáiz *et al.*, 2006), it implies that it is at least indirectly involved in interdomain communication.

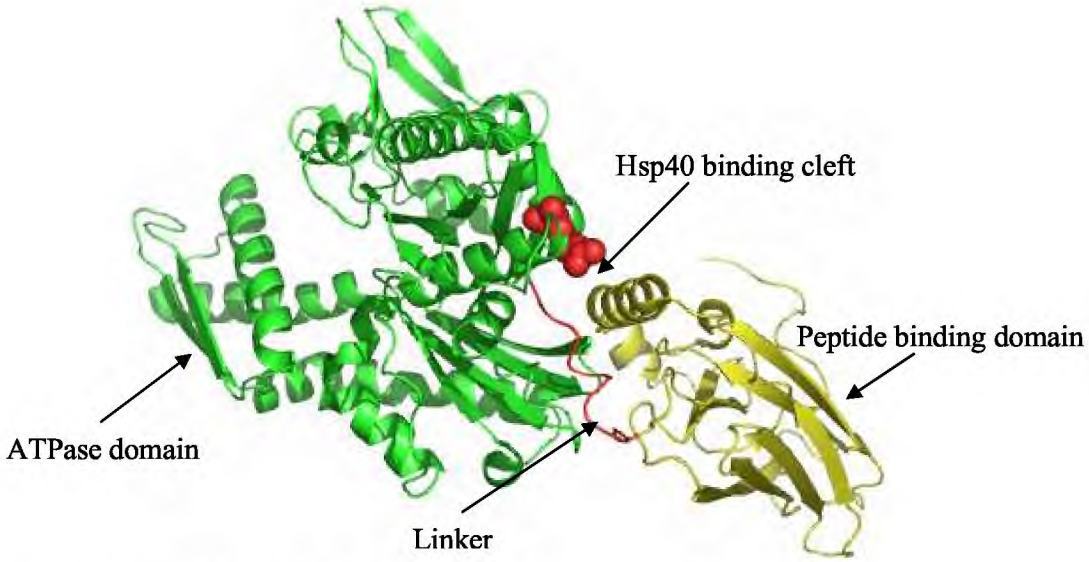

Figure 1.5 Hsp70 interdomain interface
The ATPase domain (residues 1-383; green) is linked to the peptide binding domain (residues 395-554; yellow colour) through a highly conserved linker region (residues 384-394; in red colour). The proximity of the peptide binding and ATPase domains is highlighted by residue R171 in space fill (red) which is believed to be important for Hsp40 interaction with Hsc70 (Suh *et al.*, 1998; Jiang *et al.*, 2005). As the J domain of Hsp40 accesses its binding cleft in the Hsc70 ATPase domain, it is thought to get in physical contact with the peptide binding domain of Hsc70 (Jiang *et al.*, 2005). The figure of Hsc70 (1YUW.pdb; Jiang *et al.*, 2005) was visualised using PyMol (DeLano, 2002).

The role of ATP and ADP in the chaperone activity of Hsp70 and in particular interdomain communication of the protein has recently been in the spotlight (Swain *et al.*, 2006). Traditionally, it is believed that Hsp70 in the ATP bound state has less affinity for the substrate than in the ADP bound state (Suh *et al.*, 1999). Swain *et al.* (2006) whilst acknowledging the role of ATP in reducing Hsp70 substrate affinity, question the role of the substrate in influencing events in the ATPase domain. A peptide bound to a nucleotide free DnaK only induced localised intradomain chemical shifts in the peptide binding domain that did not transcend to the linker region (Swain *et al.*, 2006). This study proposed that in the absence of ATP, the ATPase and peptide binding domains of Hsp70 do not interact in the absence or presence of peptide (Swain *et al.*, 2006). Swain *et al.* (2006) attributed the previously observed global chemical shifts induced by peptide on Hsp70 to be due to the fact that in all such case studies (Jordan and McMacken, 1995; Slepenkov and Witt, 1998;

Slepenkov and Witt, 2003), ATP was bound to Hsp70. Furthermore, it was noted that in the absence of nucleotide and indeed in the ADP bound state, the peptide binding domain of DnaK is always in a state of high substrate affinity (Swain *et al.*, 2006). This study therefore strongly argues for the mandatory presence of ATP in the ATPase domain for the allosteric interaction of DnaK to occur.

1.4.6.2.6 Hsp70 and its intersectional role as a molecular chaperone

Hsp70 has a central chaperone role through its close links with other chaperones and co-chaperones (Borges and Ramos, 2005). In this way, Hsp70 serves as the intersectional chaperone by receiving unfolded proteins and then passing them on to other chaperones. DnaK is involved in reciprocal exchange of peptide substrates with GroEL (Buchberger *et al.*, 1996). In fact in *E. coli*, molecular chaperones such as DnaK, DnaJ, GrpE and GroEL/GroES have overlapping functions and the overproduction of one tends to compensate for the other (El Hage *et al.*, 2001).

It is intriguing that DnaK is dispensable in *E. coli* despite its close association with nascent peptide chains coming off the ribosomes (Teter *et al.*, 1999). The fact that another chaperone, trigger factor, can bind nascent polypeptides (Stoller *et al.*, 1995) has shed insight to this paradox. Interestingly, neither DnaK nor trigger factor (Guthrie and Wickner, 1990) is essential for growth, but mutation of both proteins results in cell death. It has since been established that the two chaperones have an overlapping role (Teter *et al.*, 1999; Deuerling *et al.*, 1999).

A functional partnership between DnaK and ClpB (Hsp100 homologue) has been observed (Diamant *et al.*, 2000). In this network, DnaK is thought to reverse small aggregates more readily than it could disaggregate larger ones, and therefore requires the intervention of ClpB to solubilise the large aggregates satisfactorily (Diamant *et al.*, 2000). Therefore, it appears that DnaK curtails misfolding and aggregation of proteins that are early victims of misfolding. ClpB only becomes essential when protein aggregation occurs beyond what DnaK is able to manage and ClpB intervenes by keeping the aggregates in a form that allows them easy recognition by DnaK (Kedzierska and Matuszewska, 2001). Hsp70 also co-operates with small heat shock proteins in reversing aggregation (Frydman *et al.*, 1994). In

fact *E. coli* small heat shock proteins, ClpB and DnaK all cooperate in forming a functional triad that reverses protein aggregation (Mogk *et al.*, 2003).

The exchange of substrates between Hsp70 and Hsp90 has been documented (Pearl and Prodromou, 2001; Wegele *et al.*, 2004). Although both Hsp70 and Hsp90 have independent chaperone roles, it has been observed that some peptide substrates are passed from Hsp70 to Hsp90 (Wegele *et al.*, 2004). Hsp70/Hsp90 organizing protein (Hop) has been identified as playing the adaptor role, functionally linking Hsp70 to Hsp90 (Smith *et al.*, 1993). Most of the peptide substrates that have been implicated in the Hsp70/Hsp90 partnership are steroid hormone receptors and kinases. Cooperation between Hsp70 and Hsp90 in the presence of Hop during the processing of a glucorticoid receptor from a non-steroid form to its steroid state has been observed *in vitro* (Dittmar and Pratt, 1997). A model to explain how these two chaperones cooperate in the management of their client peptide substrate has been proposed (Wegele *et al.*, 2004). In the first step, Hsp70 binds the peptide in its typical operational fashion with the aid of Hsp40 protein. Another co-chaperone of Hsp70, Hsc70 interacting protein (Hip; Nelson *et al.*, 2004), joins the complex, giving it stability. It is also believed that Hip speeds up the maturation of the steroid receptor precursor (Nelson *et al.*, 2004). The second step involves Hop bringing Hsp70/substrate complex into contact with Hsp90 (the intermediate stage). Finally, Hop, Hsp70 and its co-chaperones Hsp40 and Hip are released, with the resultant formation of a mature complex that is stabilised by the presence of p23 (Morishima *et al.*, 2003).

1.4.6.2.7 Hsp70 as thermosensor

During stress, Hsp70 undergoes increased phosphorylation (Sherman and Goldberg, 1993). Phosphorylation of Hsp70 influences its interaction with substrates and co-chaperone, regulating its physical characteristics such as dimerisation (Sherman and Goldberg, 1993). In DnaK, threonine-199 is the target of phosphorylation and for this reason this residue is important for the regulation of the ATPase activity of DnaK (McCarty and Walker, 1991). The thermoregulation of both the ATPase activity and phosphorylation of DnaK has led to speculation that DnaK is a physiological thermosensor (McCarty and Walker, 1991). As a thermosensor, DnaK ensures that cells survive environmental stress not only by upregulating

DnaK's expression (Grossman *et al.*, 1987), but through an upshift of its chaperone activity as well (McCarty and Walker, 1991).

The thermoregulation of DnaK's activity justifies its role in the regulation of the heat shock response (Tilly *et al.*, 1983). In *E. coli*, DnaK is not the only important chaperone in the regulation of the heat shock response since DnaJ and GrpE also play the same role (Straus *et al.*, 1990). The endoplasmic-based chaperone calreticulin has also been observed to increase its ability to bind peptide substrates at higher temperatures (Rizvi *et al.*, 2004). GrpE is a thermosensor in its own right and employs its two long helix arms to regulate nucleotide exchange depending on the physiological state of the cell (Grimshaw *et al.*, 2003). For example, during heat stress, it slows down nucleotide exchange cycles, keeping DnaK in the ADP state for longer periods of time, thus ensuring that a higher fraction of peptide substrate is bound to DnaK (Siegenthaler *et al.*, 2004). Interfering with the role of the long helix pair through disulphide bond stabilisation resulted in compromised protein folding during heat shock (Grimshaw *et al.*, 2003). The fact that GrpE is the only one that is essential at all temperatures (Ang and Georgopoulos, 1989) in the DnaK/DnaJ/GrpE chaperone system is testimony to its importance as a thermosensor in *E. coli* (Siegenthaler and Christen, 2005). It is not surprising that the physiological levels of GrpE are closely regulated at all times (Brehmer *et al.*, 2004).

1.5 Heat shock proteins in health and disease

In light of the central role heat shock proteins play in the cell, it comes as no surprise that they have been implicated in disease development (Young, 1990). However, the role of heat shock proteins in disease development is marred with controversy. For example, one study suggested that over-expression of Hsp70 led to improved cell survival against Fas induced apoptosis (Schett *et al.*, 1999), while another one established that over-expression of the chaperone supported apoptosis (Beere *et al.*, 2000). The potential use of heat shock proteins as an indicator of wellness has been proposed (Terry *et al.*, 2004). It has been suggested that a low level of circulating Hsp70 is a potential indicator of wellness and that the level of this chaperone could be used as a pointer to longevity (Terry *et al.*, 2004). However, there is evidence that Hsp70 expression declines in normal aging cells of primate retina (Bernstein *et*

al., 2000). Heat shock proteins are induced during graft tissue preservation and during surgery, leading to speculation that they promote graft rejection (Baba, 1997).

Heat shock proteins of pathogenic origin that are produced during host invasion elicit antibody production in host cells (Maresca and Kobayashi, 1994; Zügel and Kaufmann, 1999) and some of these heat shock proteins have become targets of vaccine research (Newport, 1991). Because of their ubiquity and conservation, it has been proposed that heat shock proteins are at the interface between infection and autoimmunity through the recognition of their conserved epitopes, thus promoting cross reactivity (Lamb *et al.*, 1989; Zügel and Kaufmann, 1999). On a positive note, heat shock proteins play an important role during the presentation of antigen to the antibodies (Vanbuskirk *et al.*, 1989: Zügel and Kaufmann, 1999). Recent evidence that links heat shock proteins with drug resistance in fungus has been unearthed, and both Hsp90 (Cowen and Lindquist, 2005) and its functional partner, calcineurin (Sanglard *et al.*, 2003), were implicated.

Parasitic organisms produce heat shock proteins in response to stress as well as for developmental purposes (Silva *et al.*, 1998; Feder and Hofman, 1999). It has been suggested that expression of these proteins during parasitic development facilitates smooth adaptation during growth from one developmental phase to another (Silva *et al.*, 1998). During invasion, parasites express heat shock proteins in order to adapt to change, whilst host cells express heat shock proteins as a defensive mechanism (Schlesinger, 1990). The expression of heat shock proteins by parasites during invasion introduces an interesting paradox. On one end, it benefits the parasite in its bid to infect the host by providing the chaperone machinery, which in turn supports its ability to express essential, properly folded proteins. On the other hand, the expressed heat shock proteins serve as an early warning signal to the host's immune system alerting it to the presence of the parasite. A study suggested that the host cell was the net beneficiary from an immunological point of view during the chronic phase of infection by *Mycobacterium tuberculosis* (Stewart *et al.*, 2001). This is because the expression of Hsp70 by the parasite does not only signal the presence of the parasite, but also facilitates the presentation of other parasitic proteins to the host, thus eliciting an immune response that targets all antigenic proteins from the parasite besides its heat shock proteins (Stewart *et al.*, 2001).

1.6 Heat shock proteins in the life cycle of *Plasmodium* species

1.6.1 Malaria and the life cycle of *Plasmodium* species

Malaria is rated as the worst parasitic disease affecting 107 countries and a population of 3.2 billion people (40% of the world's population) (Thiel, 2005). Clinical cases for malaria range between 350 – 500 million of which 1 – 2 million result in deaths annually (Thiel, 2005). Four species of *Plasmodium* are responsible for causing the disease and these are: *P. falciparum*, *P. vivax*, *P. malaria*, and *P. ovale*. Of these, it is *P. falciparum* that accounts for the highest mortality rate (Thiel, 2005). However, though acounting for less malaria mortality rate, *P. berghei* and *P. knowlesi* are also known to infect humans (Franz *et al.*, 1987; Jongwutiwes *et al.*, 2004.). Therefore, it is falciparum malaria that has received a lot of research attention. The malaria parasite's life cycle is complex and dynamic, transcending across the cold-blooded mosquito vector and the vertebrate host (Figure 1.6).

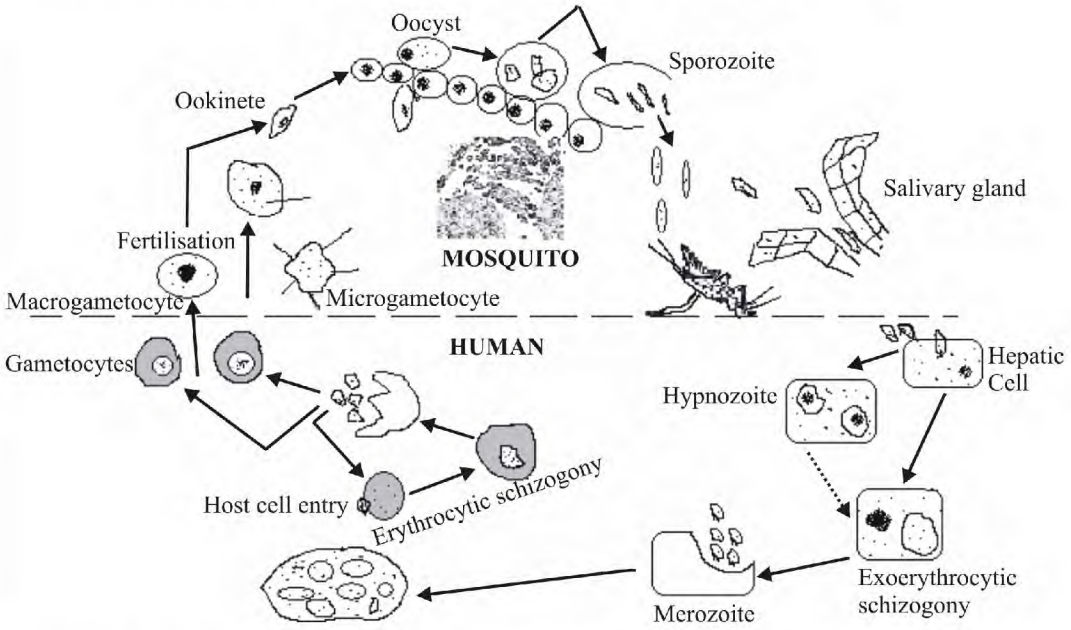

Figure 1.6 Life cycle of malaria parasites
When female mosquitoes bite the vertebrate host, they transmit the sporozoites into the host (Fujioka and Aikawa, 2002). Within a short time the sporozoites enter the hepatocytes. Within 5 – 15 days, depending on the *Plasmodium* species, the schizont stage is attained (Fujioka and Aikawa, 2002). *P. vivax*, *P. ovale* and *P. cynomolgi* go through a dormant (hypnozoite) stage that may last for weeks or many years before developing into preerythrocytic schizogony (Cogswell, 1992). This causes malaria relapses (Cogswell, 1992). The preerythrocytic schizont contains 10 000 – 30 000 merozoites which are released into the blood circulatory system and invade red blood cells. Once in the red blood cell, the merozoite goes through the ring, trophozoite and schizont stages (erythrocytic schizogont). Part of the parasitic population differentiates into sexual forms – macrogametocyte (female) and microgametocyte (male). The mosquito then takes up the mature sexual forms. Microgametocytes become flagellated into exflagetting microgametocyte. The sexual forms then fertilise to form a zygote. The non-motile zygotes develop into motile ookinetes, which move to reach the epithelium of the midgut before transforming into oocyst. Within 10 – 24 days after infection sporozoites enter the salivary gland for retransmission into the host again (adapted from Fujioka and Aikawa, 2002).

1.6.2 *Plasmodium falciparum* heat shock response

Plasmodium falciparum experiences physiological challenges associated with its passage from the cold-blooded mosquito vector to the warm-blooded human host. Once in the human host, it has to contend with the additional heat stress emanating from the development of malaria fever. For this reason, the parasite's genome is largely committed to immune evasion and host-parasite invasion (Gardner *et al.*, 2002). The production and localisation of thermo-inducible heat shock proteins in *P. falciparum* has been well documented (Kumar *et al.* 1991; Joshi *et al.* 1992; Biswas and Sharma 1994; Watanabe 1997). There is growing evidence that heat shock proteins from *P. falciparum* could play a cytoprotective role in the life cycle of the parasite. The importance of chaperones at the host-parasite interface as a survival strategy has been reviewed (Feder and Hofman, 1999). Furthermore, chaperones are the main protein components apart from proteases in the parasitophorous vacuole of *P. falciparum* (Nyalwidhe and Lingelbach, 2006). This suggests that quality control of the *P. falciparum* proteosome is an important aspect during invasion judging from the abundance of these two groups of proteins, both of which have a role to play in the life cycle of proteins.

The induction of major molecular chaperone families in *Plasmodium* species is directly linked to their phosphorylation (Wiser and Plitt, 1987; Kappes *et al.*, 1993). The same phenomenon is true for *E. coli* DnaK whose temperature dependent expression is associated with maximum phosphorylation (McCarty and Walker, 1991). This ensures that not only is the Hsp70 protein abundantly expressed in the event of stress, but that its chaperone activity is enhanced (McCarty and Walker, 1991). The concomitant expression and phosphorylation of PfHsp70s in *P. falciparum* has been observed (Kappes *et al.*, 1993) and this could be a way of regulating its chaperone activity. A homologue of p23 and hip from *P. falciparum* is expressed and undergoes phosphorylation during the development of the parasite (Wiser, 2003). The existence of this protein in the parasite has been linked to a potential p23-PfHsp90 partnership (Kumar *et al.*, 2003), which is important in the establishment of the steroid hormone receptor complexes (Richter and Buchner, 2001). A cytosolic phosphoprotein and homologue of Hip/p48 with a GGMP motif from *P. berghei*, has also been identified (Wiser *et al.*, 1996).

1.6.3 Major groups of heat shock proteins from *Plasmodium falciparum*

1.6.3.1 *Plasmodium falciparum* Hsp90

PfHsp90 is essential for the survival of *P. falciparum* and has been found to occur in multi-protein complexes with other parasitic chaperones and co-chaperones (Banumathy *et al.*, 2003). One of the proteins that have been found to be closely associated with PfHsp90 is calneurin and it is deemed to require PfHsp90 for its folding (Kumar *et al.*, 2005). Interestingly, geldanamycin an antimalarial inhibitor of PfHsp90 (Banumathy *et al.*, 2003; Kumar *et al.*, 2003) was found to synergistically support the antiplasmodial activity of cyclosporin A, a known inhibitor of eukaryotic calneurin (Milan *et al.*, 1994). Another phosphatase from *P. falciparum*, PfPP5, is expressed at the erythrocytic stage and closely associates with PfHsp90; perhaps acting as PfHsp90's co-chaperone (Dobson *et al.*, 2001). Therefore, there is growing evidence that chaperones from *P. falciparum* are important for its survival. However, it should be noted that it seems that the parasite exploits host cell chaperones during the assembly of multi-protein subunits at the erythrocyte surface (Banumathy *et al.*, 2002). Host Hsp70 and Hsp90 in red blood cells that were infected with the parasite were membrane associated whereas the same chaperones were largely found in soluble, cytosolic forms in uninfected cells (Banumathy *et al.*, 2002). This suggests that in infected cells, host chaperones could be facilitating the trafficking of parasitic proteins to the surface of the red blood cell (Banumathy *et al.*, 2002).

During the development of febrile malaria, body temperature rises to 41°C due to the release of proinflammatory cytokine tumour necrosis factor (Karnumaweera *et al.*, 1992) and the development of this fever is known to promote the pathogenesis of malaria (Udomsangpetch *et al.*, 2002). This is because initial heat shock prepares the parasite for better thermal resilience against successive heat shock (Pavithra *et al.*, 2004). Consequently, febrile episodes that are synonymous with the development of malaria tend to promote intra-erythrocytic development of the parasite, enhancing circulation counts, hence boosting pathogenesis (Pavitra *et al.*, 2004). This phenomenon has been observed in other protozoan species (Soete *et al.*, 1994; Wiesgigl and Clos, 2001). PfHsp90 in particular, is thought to play an important role to ensure the development and survival of *P. falciparum* during the intra-erythrocytic phase (Pavinthra *et al.*, 2004). The role of Hsp90 acting at the crossroads between development and environment has been observed in other protozoa (Wiesgigl and

Clos, 2001), further highlighting its role as a 'capacitor' for morphological development (Sollars *et al.*, 2003).

1.6.3.2 Small heat shock proteins from *Plasmodium* species

Small heat shock proteins are known to be important for disaggregation and refolding of denatured proteins (Jakob *et al.*, 1993). *P. falciparum* polyubiquitin (PfUB), a small heat shock protein, which is subject to both heat shock and growth phase induction has been identified (Horrocks and Newbold, 2000). *P. vivax* also expresses a small heat shock protein at its erythrocytic stage of development and the protein has metalloprotease activity (Fakruddin *et al.*, 2000).

1.6.3.3 *Plasmodium falciparum* Hsp40 proteins

The earliest attempt at characterising Hsp40 proteins from *P. falciparum* came from work done by Watanabe (1997) who characterised PfJ1-4. According to the DnaJ canonical classification system (Cheetham and Caplan, 1998), PfJ1 is a type I Hsp40, PfJ4 is a type II, whilst PfJ2 and PfJ3 are type III Hsp40s. Heat shock evoked a sharp increase in steady state PfJ3 mRNA levels, whilst a reduced response was observed for PfJ1 and PfJ4. On the other hand, PfJ2 induction was reduced upon heat shock (Watanabe, 1997). Another type III Hsp40-like protein, ring-infected erythrocyte surface antigen (RESA; Favaloro *et al.*, 1986) is produced by the parasite just before the merozoite stage of development and eventually associates with the membrane of the newly invaded red blood cell bound to the erythrocyte protein spectrin (Foley *et al.*, 1991). It shares 39% homology with *E. coli* DnaJ over a stretch of 70 amino acids (Bork *et al.*, 1992b) and the two proteins share significant homology based on minimum standards for structural homology of globular proteins (Sander and Schneider, 1991). Instead of the HPD motif that occurs in most other Hsp40-like proteins where it is crucial for their function, RESA has a YPY motif instead (Bork *et al.*, 1992b; Watanabe, 1997).

A sequence that is termed the *Plasmodium* export element (PEXEL; Marti *et al.*, 2004) motif or vacuolar transport signal (VTS; Hiller *et al.*, 2004) has been identified and directs parasite proteins to the erythrocyte (Hiller *et al.*, 2004). Based on the presence of export signals, three

type II and 16 type III Hsp40-like proteins (including RESA) are predicated to be exported (Templeton and Deitsch, 2005; Sargeant *et al.*, 2006). Type I PfHsp40-like proteins lack the export signal motif, hence are proposed to be resident in the parasite (Sargeant *et al.*, 2006). The lack of coherent overlap between proteins predicted to be exported based on the PEXEL and VTS algorithms (Hiller *et al.*, 2004; Marti *et al.*, 2004) further supports that export involves a multi-step pathway (Horrocks and Muhia, 2005; Templeton and Deitsch, 2005). In their model, Templeton and Deitsch (2005), suggested that based on the presence of the signal peptide sequence encoded by an upstream exon 1 (Marti *et al.*, 2004; Sargeant *et al.*, 2006), proteins meant for export are channelled to the parasitophorous vacuole (PV) from the ER/Golgi Apparatus system of the cell. Transport across the PV to the red blood cell then depends on the presence of PEXEL/VTS motif, which is encoded by exon II (Marti *et al.*, 2004; Templeton and Deitsch, 2005; Sargeant *et al.*, 2006). Although direct evidence to confirm the export of *P. falciparum* Hsp40 into the red blood cell is scanty, it was recently demonstrated that a GFP tagged PfHsp40 protein carrying the export predictive motif constituted of 11 amino acid residues (TSLRSLAEFNS) was able to migrate into an infected red blood cell (Hiller *et al.*, 2004). This export motif directs proteins from the *P. falciparum* vacuole to the human erythrocyte (Hiller *et al.*, 2004).

Based on the canonical Hsp40-like protein classification system, type I and II proteins generally act as co-chaperones, whilst the role of the type III group is not definitive (Walsh *et al.*, 2004). Because most of the PfHsp40s that have export motifs do not have a conserved HPD motif, which is crucial for co-chaperone function of Hsp40 proteins, the role of these proteins is not clear. Nevertheless, it is speculated that these proteins are possibly exported to the red blood cells to modulate the function of host chaperones (Sargeant *et al.*, 2006). Indeed, ATP dependent associations between host chaperones (Hsp70, Hsp60 and Hsp90) with some *P. falciparum* proteins that are exported to the red blood cell have been observed (Banumathy *et al.*, 2002).

1.6.3.4 *Plasmodium falciparum* Hsp60

Very little work has been done on the Hsp60 chaperone system of *P. falciparum*. Steady state accumulation of mRNA transcripts of PfHsp60 in response to heat shock has been observed (Syin, and Goldman, 1996). However, there was no increase in the level of the protein suggesting that regulation of expression of the PfHsp60 is either at the translational level or subject to protein stability (Das *et al.*, 1997). Characterisation of PfHsp60 showed that it co-immunoprecipitates with a host of other proteins (Das *et al.*, 1997).

1.6.3.5 PfHsp70 and its homologues from *Plasmodium falciparum*

The existence of *PFHSP70* genes on the *P. falciparum* genome has been documented and altogether, up to six Hsp70-like protein sequences have been identified (Table 1.2; Peterson *et al.*, 1988; Sargeant *et al.*, 2006). Of the six Hsp70s from *P. falciparum*, it is only PfHsp70 (Table 1.2) that has received widespread research attention in its respect as a chaperone (Sharma, 1992; Matambo *et al.*, 2004; Ramya *et al.*, 2006) and vaccine candidate (Behr *et al.*, 1992; Kumar *et al.*, 1990). In fact its heat inducibility (Joshi *et al.*, 1992) and high homology to *Drosophila* Hsp70 resulted in it being named 'PfHsp70' (Sharma, 1992). Table 1.2 is a summary of the names and features of the six *P. falciparum* Hsp70s.

Bioinformatics data also confirmed that the *PFHSP70* gene is among some of the most highly expressed genes in *P. falciparum* (Patankar *et al.*, 2001). The expression of PfHsp70 and its homologue, PfGrp78/PfHsp70-2 (PF10875w; Table 1.2), at the blood stages of the parasite has been confirmed and both proteins have been reported to be soluble (Bianco *et al.*, 1986; Sharma, 1992; Kappes *et al.*, 1993). PfHsp70 is localised in the cytoplasm and the nucleus, whilst PfHsp70-2 occurs in the ER (Kappes *et al.*, 1993). However, PfHsp70 has been reported to be present in the parasitophorous vacuole (Nyalwidhe and Lingelbach, 2006). It has been detected in the Maurer's cleft (Vincensini *et al.*, 2005), a structure that is connected to the parasite but occurs in the red blood cell (Langreth *et al.*, 1978). This has raised the possibility that this protein could be exported to the red blood cell.

Information on the chaperone role of PfHsp70 in *P. falciparum* is scanty, with most studies having mainly confirmed its heat-inducibility (Kumar *et al.*, 1991; Sharma, 1992; Biswas and Sharma, 1994). However, there is evidence that points to its possible role in actin

polymerisation (Tardieux *et al.*, 1998). Actin polymerisation is a phenomenon that implicates the role of actin filaments in facilitating host cell invasion by parasites (Dobrowolski *et al.*, 1997). It has been suggested that an Hsp70 from *P. falciparum* is involved in regulating actin polymerisation (Tardieux *et al.*, 1998). In addition, there is evidence suggesting that protein trafficking into the apicoplast of *P. falciparum* requires the role of Hsp70 proteins (Foth *et al.*, 2003).

Table 1.2 Hsp70s from *Plasmodium falciparum*

Name of Hsp70 used in this study (bold), alternate name(s) and PlasmoDB annotation

Features	**PfHsp70**[b] PfHsp[e,h] PfHsp70-1[a] PF08_0054	**PfHsp70-2**[b,c] PfGrp78[d,i] PF10875w	**PfHsp70-x** - MAL7P1.228	**PfHsp70-3**[b] PfmtHsp70[e] PF11_0351	**PfHsp70-z** - PF07_0033	**PfHsp70-y** - MAL13P1.540
Approximate molecular mass (kDa)	74	73	76	73	100	108
Chromosome	8	9	7	11	7	13
Cellular location	nucleus and cytoplasm[d]	ER[d]	cytoplasm[f] N.C	mitochondrion[e,f] N.C	cytoplasm[f] N.C	ER[f] N.C
Signal Sequence/Special feature	-C-terminal EEVD motif -GGMP motif[b]	-homologue of Bip/Grp78[d,g] -Carboxy terminal ER sequence[d]	C-terminal EEVN motif	-homologue of mtHsp70[e,f] -mitochondrial transit sequence[e,f]	homologue of cytoplasmic Hsp105[f]	Carboxy terminal ER sequence[f]
Expression phase	Exoerythrocytic stage[b]	Exoerythrocytic stage[b]	N.E	N.E	N.E	N.E

N.E (not yet established), N.C (not experimentally confirmed) and ER (Endoplasmic Reticulum)
The letters in superscript represent the following references: a-Biswas and Sharma, 1994; b-Sharma, 1992; c-Nyalwidhe and Lingelbach, 2006; d-Kumar *et al.*, 1991; e- Šlapeta and Keithly, 2004; f-Sargeant *et al.*, 2006; g-Kumar and Zheng, 1992

1.7 Research Hypothesis

There is growing speculation that PfHsp70 confers a chaperone cytoprotective role to *P. falciparum* during its life-cycle (Sharma, 1992). In addition, PfHsp70 has been found to interact with PfHsp90 in a potential multi-chaperone network (Banumathy *et al.*, 2003). To gain insight into the possible structural-functional features of this protein, its overproduction in *E. coli* and partial characterisation has been conducted (Matambo *et al.*, 2004; Ramya *et al.*, 2006). However, there is need for further analysis of the chaperone function of this protein, such as its interaction with co-chaperones and peptide substrate. Previous studies on the characterisation of the protein have been based on the use of protein purified through denatured purification with native elutions (Matambo *et al.*, 2004; Ramya *et al.*, 2006) and this study covers attempts made in order to purify the protein natively for use *in vitro* studies. In this study, it is hypothesised that PfHsp70 is a chaperone and should be able to display typical Hsp70 chaperone features both *in vivo* and *in vitro*. Thus, PfHsp70 should be able to not only display chaperone properties on its own, but must have its chaperone activity regulated by co-chaperones from *P. falciparum*.

Based on this hypothesis, the following are the broad objectives of this research:

1. Conducting bioinformatics studies aimed at gleaning the chaperone features of PfHsp70.
2. To investigate the chaperone role of PfHsp70 using an *in vivo* assay.
3. Analysing PfHsp70 chaperone properties using *in vitro* assays.
4. Characterisation of the structural features important for PfHsp70 function such as the peptide binding domain and the linker region of the protein.
5. Investigate whether PfHsp70's ATPase activity is modulated by an Hsp40 protein from *P. falciparum* (e.g. PfJ4).

Work covered in chapter 2 involved bioinformatics studies conducted in order to establish the structural motifs of PfHsp70 that distinguishes it as a unique Hsp70 as well as identify features that it shares with other Hsp70 homologues. A comparative analysis of PfHsp70's chaperone features relative to its *P. falciparum* homologues is also discussed in the next chapter.

Chapter Two

Bioinformatic analysis of the chaperone properties of PfHsp70 and its homologues from *Plasmodium falciparum*

2.1 Introduction

Bioinformatics is the study of biological molecules by making use of tools from various disciplines such as mathematics, statistics and computer science (informatics) (Luscombe *et al.*, 2001). The emergence of this relatively new field of research has been triggered largely due to the generation of huge volumes of information emanating from gene sequencing projects, thus necessitating the need for computational input in order to ease data management. In addition, the emergence of bioinformatics as an essential aspect of research in molecular biology is based on the rationale that life systems are sustained by reactions that are run by 'programs' encoded in genes (Luscombe *et al.*, 2001). Furthermore, beyond the gene level of organisation, biological molecules such as proteins occur in structurally distinct families that have the form of digital data sets (Luscombe *et al.*, 2001). Therefore, bioinformatics is crucial in the management of biological data, development of tools for analysing and interpreting the data (Luscombe *et al.*, 2001).

Structural studies have revealed that proteins have limited fold options despite their variation in identity (Orengo *et al.*, 1994). This is because different proteins have multiple copies of structural motifs that restrict their fold options even if they share remote identities (Leak and Chothia, 1980). This has led to the development of tools enabling researchers to glean possible functional roles of unknown proteins based on the conformity of their amino acid sequences to a protein whose structure and function is well established (Zvelebil *et al.*, 1987). However, occasionally, fold variation of notable magnitudes is observed in proteins that may share high sequence similarity (Russel and Barton, 1993).

Plasmodium falciparum belongs to the apicomplexan species of organisms. These are organisms that have a chloroplast-like organelle (apicoplast) of no photosynthetic significance (Blanchard and Hicks, 1999). The apicoplast hosts metabolic pathways that are distinct from those of the host cell making it a target for drug design (Ralph *et al.*, 2001). It has been established that Hsp70s of apicomplexan origin share phylogenetic links (Šlapeta

and Keithly, 2004). The mitochondrial and cytolosolic Hsp70 homologues from the apicomplexan kingdom and other closely related species display distinct phylogenetic features, suggesting that these proteins have distinct roles (Šlapeta and Keithly, 2004). At least six *P. falciparum* Hsp70 homologues with features spanning across the cytosolic, endoplasmic reticulum (ER), and the mitochondrial forms have been identified (Table 1.2; Sargeant *et al.*, 2006). Of these only PfHsp70 and PfHsp70-2 (Table 1.2) have been partially characterised (Kumar *et al.*, 1991; Kappes *et al.*, 1993). Only PfHsp70 was significantly induced in response to heat stress (Kumar *et al.*, 1991). Heat stress only led to a slightly elevated expression of PfHsp70-2 (Kumar *et al.*, 1991). The subcellular localisation of these proteins differs; PfHsp70-2 is localised to the cytoplasm in ER-like structures, whilst PfHsp70 occurs in the cytoplasm and nucleus (Kumar *et al.*, 1991).

Hsp70s from *P. falciparum* and another parasite, *Trypanosoma cruzi* have display closely related expression profiles and functional features (Kumar *et al.*, 1991; Kappes *et al.*, 1993; Olson *et al.*, 1994; Edkins *et al.,* 2004; Matambo *et al.*, 2004). The cytosolic and mitochondrial Hsp70 homologues from *Trypanosoma cruzi* are induced in response to heat stress (Olson *et al.*, 1994). However, of these two *Trypanosoma cruzi* Hsp70 homologues, only the cytosolic Hsp70 protein goes into the nucleus in response to heat stress (Olson *et al.*, 1994). This scenario is comparable to that observed in *P. falciparum* where only the cytosolic Hsp70 moves into the nucleus whilst the ER homologue remains in the cytoplasm when *P. falciparum* is exposed to heat stress (Kappes *et al.*, 1993). During growth-related development from proliferation to differentiation, the levels of cytosolic *Trypanosoma cruzi* Hsp70 decreased drastically, whilst that of the mitochondrial Hsp70 fell mildly (Olson *et al.*, 1994). Altogether, this evidence further supports that Hsp70 homologues of parasitic origin have specialised and localised function. Another feature that seems to characterise Hsp70s from *Trypanosoma cruzi* and *P. falciparum* is the generally high basal ATPase activity that they display (Olson *et al.*, 1994; Edkins *et al.,* 2004; Matambo *et al.*, 2004). Perhaps this suggests that Hsp70s of parasitic organisms have distinct functional features compared to their homologues from other free-living eukaryotic organisms.

The use of protein homology has become a useful tool in predicting protein function (Russel and Barton, 1993). This is because the three dimensional folds of proteins are predictably similar or different depending on their degree of sequence conservation (Zvelebil *et al.*, 1987). Since Hsp70s are highly conserved across species, they display conserved features

that are important for their chaperone role. These include key residues for interaction with their Hsp40 partners, NEF, and peptide substrate. It would be envisaged that PfHsp70 must possess structural features that qualify it to be a molecular chaperone based on its structural resemblance to other well characterised Hsp70s from prokaryotic and eukaryotic kingdoms. Therefore, the main objective of this study was to analyse PfHsp70 chaperone properties embedded in its amino acid sequence. Insight on the chaperone features of PfHsp70 was established by comparing its sequence features to the same structural details in *E. coli* DnaK and human Hsc70. In addition, the comparison of structural features of PfHsp70 to human Hsc70 could be used to identify potential structural-functional features whose variation could be exploited in the development of potential inhibitors targeting PfHsp70 without affecting Hsc70. For example, Matambo *et al.* (2004) established that while PfHsp70 had a high basal ATPase activity compared to human Hsc70, it had a lower affinity for ATP. This suggests that limiting cellular levels of ATP could interfere selectively with PfHsp70 function, without affecting human Hsc70 function (Matambo *et al.*, 2004). This paves the way for the possible development of PfHsp70 inhibitors that may not interfere with Hsc70 function.

The broad objectives of this study were to:

1) Analyse the sequence similarity between PfHsp70, *E. coli* DnaK and human Hsc70.
2) Investigate the chaperone features of PfHsp70 relative to other *P. falciparum* Hsp70 homologues.
3) Identify putative residues that could play a role in the interaction of PfHsp70 and its potential Hsp40 partners, NEFs and its peptide substrates.
4) Carry out phylogenetic analysis of *P. falciparum* Hsp70 proteins relative to their homologues from other species.
5) Identify peptide signal motifs that are important in predicting the localisation of *P. falciparum* Hsp70 proteins.
6) Identify *P. falciparum* proteins that potentially interact with *P. falciparum* Hsp70s.

2.2 Experimental Procedures

2.2.1 Comparative investigation of the phylogenetic and chaperone features of PfHsp70 and its *Plasmodium falciparum* homologues

Using phylogenetic and sequence analysis tools, the chaperone properties of PfHsp70 were studied in relation to Hsp70s from other eukaryotic and prokaryotic species. The phylogenetic profile of *P. falciparum* Hsp70s in relation to Hsp70s from other species was established based on amino acid sequence comparisons. The *P. falciparum* Hsp70 protein sequences (Table 1.2) were downloaded from PlasmodDB (www.PlasmoDB.org). The rest of the protein sequences were downloaded from the National Center for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov). Multiple sequence alignments were carried out using the BioEdit (www.mbio.ncsu.edu/BioEdit/bioedit.html) programme based on the ClustalW alignment tool (Thompson *et al.*, 1994). The phylogenetic dendrograms were generated using the BioEdit based Protdist – neighbour phylogenetics tree option and displayed by TreeView software application (Page, 1996).

2.2.2 Protein homology modelling

Protein homology models were generated using SWISS-MODEL, first approach mode (Peitsch, 1995; Guex *et al.*, 1997; Schwede *et al.*, 2003). The images of the modelled proteins were visualised using the PyMol programme (DeLano, 2002).

2.3 Results

2.3.1 Analysis of the sequence similarity between PfHsp70, *E. coli* DnaK and human Hsc70

Amino acid sequence alignments of the ATPase and peptide binding domains of PfHsp70, *E. coli* DnaK and human Hsc70 were carried out (Figures 2.1 and 2.2). Generally, there is a higher degree of conservation across the ATPase domains of PfHsp70, DnaK and Hsc70 (Figure 2.1; Bork *et al.*, 1992a) than is observed for the peptide binding domains (Figure 2.2).

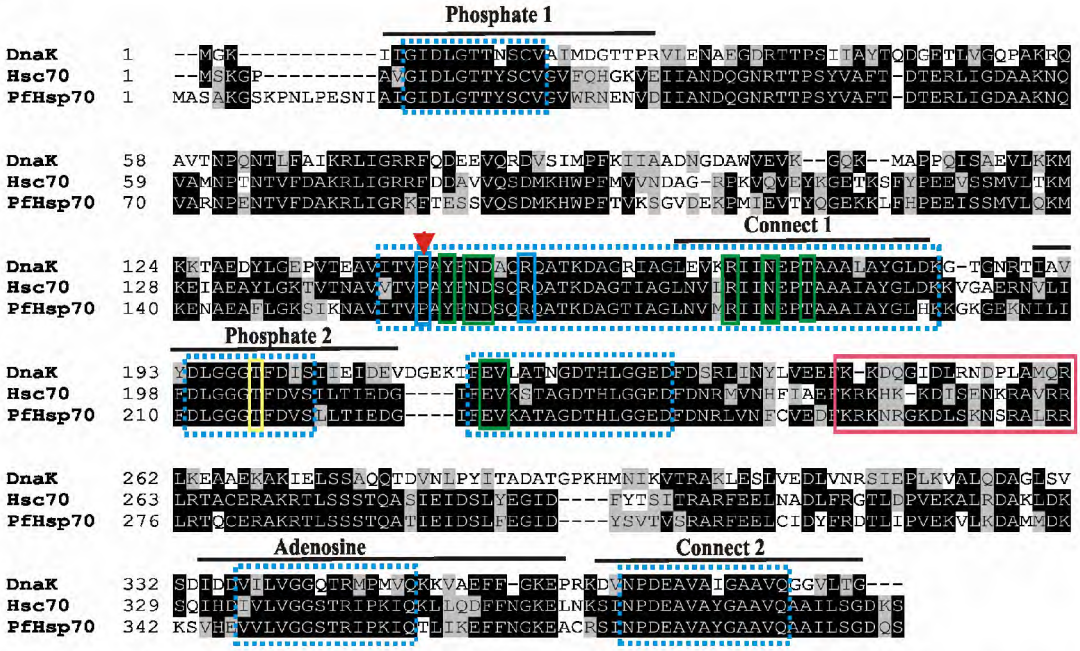

Figure 2.1 Sequence alignment of the ATPase domains of PfHsp70, *E. coli* DnaK and human Hsc70
The amino acid sequences representing the ATPase domains of *E. coli* DnaK (accession number: BAA01595.1), human Hsc70 (accession number: AF352832) and PfHsp70 (accession number: PF08_0054) were aligned using the BioEdit (www.mbio.ncsu.edu/BioEdit/bioedit.html)-ClustalW alignment tool (Thompson *et al.*, 1994). The following residues were highlighted: bipartite nuclear localisation signal (Robbins *et al.*, 1991; red box); and highly conserved residues associated with heat shock proteins (Mount, 1985; blue boxes, with broken lines). Based on studies on DnaK, some residues that are important for its function and their corresponding residues in Hsc70 and PfHsp70 are highlighted as follows: P143, proline allosteric switch, marked by red arrow; and R151 both of which are important for interdomain function (Vogel *et al.*, 2006a; blue boxes with continuous lines); Y145, N147, D148, N170 and T173 that interact with DnaJ (Gässler *et al.*, 1998; Suh *et al.*, 1998; green boxes); T199, a DnaK phosphorylation site (McCarty and Walker, 1991; yellow box). Motifs that interact with the respective units of the nucleotide (phosphate 1, phosphate 2 and adenosine) are shown (Bork *et al.*, 1992a; bold lines), including the segments that link up these subdomains ('connect 1 and 2'). Identical residues are presented in white colour (on black background), whilst similar residues are shown in black colour (on grey background).

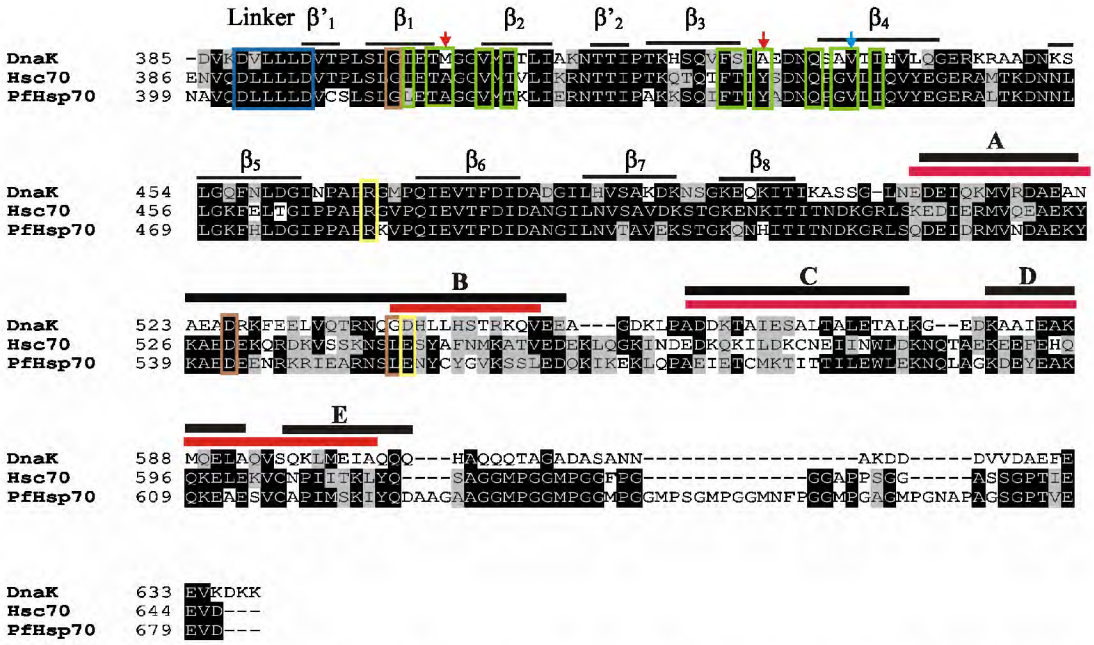

Figure 2.2 Sequence alignment of the peptide binding domains of PfHsp70, *E. coli* DnaK and human Hsc70

Sequence alignment for the peptide binding domains of *E. coli* DnaK (accession number: BAA01595.1), human Hsc70 (accession number: AF352832) and PfHsp70 (accession number: PF08_0054) were aligned using BioEdit programme (www.mbio.ncsu.edu/BioEdit/bioedit.html)-ClustalW alignment option (Thompson *et al.*, 1994). The following structural features are highlighted: the highly conserved linker segment (blue box); α-helical subdomains of *E. coli* DnaK (A-E) (Chou *et al.*, 2003; black, bold line for subdomains A-E of DnaK; and red bold line for Hsc70 subdomains A-C). The following DnaK residues are highlighted together with corresponding residues in Hsc70 and PfHsp70: residues that contact substrate in DnaK (Zhu *et al.*, 1996; green boxes). Of these, M404 and A429 constituting the arch are highlighted by the red arrows; and the V436 constituting the hydrophobic pocket in DnaK is highlighted by the blue arrow (Mayer *et al.*, 2000). In addition, residues G400, D526 and G539 in the peptide binding domain of DnaK that are important for interaction with DnaJ, and their equivalent residues in Hsc70 and PfHsp70 are shown (Suh *et al.*, 1998; brown boxes). Based on Hsc70 studies, residues E543 and R469 and their corresponding residues in DnaK and PfHsp70 (yellow boxes), form a latch that stabilises the lid (Ha *et al.*, 1997; Chang *et al.*, 2001). Identical residues are presented in white colour (on black background), whilst similar residues are shown in black (on grey background).

Amongst some of the most conserved residues across the three Hsp70 species are the proline allosteric switch (Figure 2.1; Vogel *et al.*, 2006a) and several DnaJ contact sites in DnaK housed both in the ATPase and peptide binding domains (Figures 2.1 and 2.2). As expected, there is a higher degree of sequence consensus in the β-subdomain of the peptide binding domain than in the α- helical subdomain (Figure 2.2; Zhu *et al.*, 1996). Generally, residues that occur in physical contact with the peptide substrate are conserved across the three Hsp70s (Figure 2.2; Zhu *et al.*, 1996). A structural distinction noteworthy is that DnaK has five α- helical subdomains (A-E), whilst Hsc70 has only three α- helical subdomains (A-C; Chou *et al.*, 2003). Overall, PfHsp70 is more similar to Hsc70 (69 % identitical) than *E. coli* DnaK (44 % identical).

2.3.2 Sequence analysis of *Plasmodium falciparum* Hsp70 proteins and identification of their key functional motifs

2.3.2.1 Chaperone functional features of *Plasmodium falciparum* Hsp70s

Multiple sequence alignments for all the six Hsp70 homologues from *P. falciparum* (Figures 2.3 and 2.4) showed that residues associated with heat shock proteins (Mount, 1985) are generally conserved in the rest of the proteins except for PfHsp70-y and PfHsp70-z. PfHsp70-y and PfHsp70-z share greater homology with their counterparts in the ATPase domain than in the peptide binding domain. Both of these proteins do not have a distinct linker section, despite the fact that this segment is the most highly conserved in Hsp70 proteins (Karlin and Brocchieri, 1998) and is essential for their function (Vogel *et al.*, 2006b). Both PfHsp70-y and PfHsp70-z are the only *P. falciparum* Hsp70s that lack the equivalence of the DnaK phosphorylation site (T199) (McCarty and Walker, 1991). It is also notable that there is a high degree of conservation of residues that are known to be important for interaction with Hsp40 partners (Gässler *et al.*, 1998; Suh *et al.*, 1998). However, there is less conservation of these residues in PfHsp70-y and PfHsp70-z (Figures 2.3 and 2.4).

Pairwise sequence identity analysis for *P. falciparum* Hsp70s (Table 2.1) shows a wide range of percentage identities across these proteins. The highest identity (73%) is between PfHsp70 and PfHsp70-x, which is consistent with the results from phylogenetic analysis which had the two proteins clustering very close to one another (Figure 2.5). PfHsp70-x also shares relatively high identity (54%) with the putative ER homologue, PfHsp70-2. The ER based Hsp70 (PfHsp70-2) and its mitochondrial homologue (PfHsp70-3) share 46% identity between them. The rest of the proteins share very low identity between them.

Table 2.1 Percentage identities for Hsp70s from *Plasmodium falciparum*

	PfHsp70	PfHsp70-2	PfHsp70-3	PfHsp70-x	PfHsp70-y	PfHsp70-z
PfHsp70	-	53	43	73	16	17
PfHsp70-2	53	-	46	54	18	15
PfHsp70-3	43	46	-	46	16	16
PfHsp70-x	73	54	46	-	16	19
PfHsp70-y	16	18	16	16	-	20
PfHsp70-z	17	15	16	19	20	-

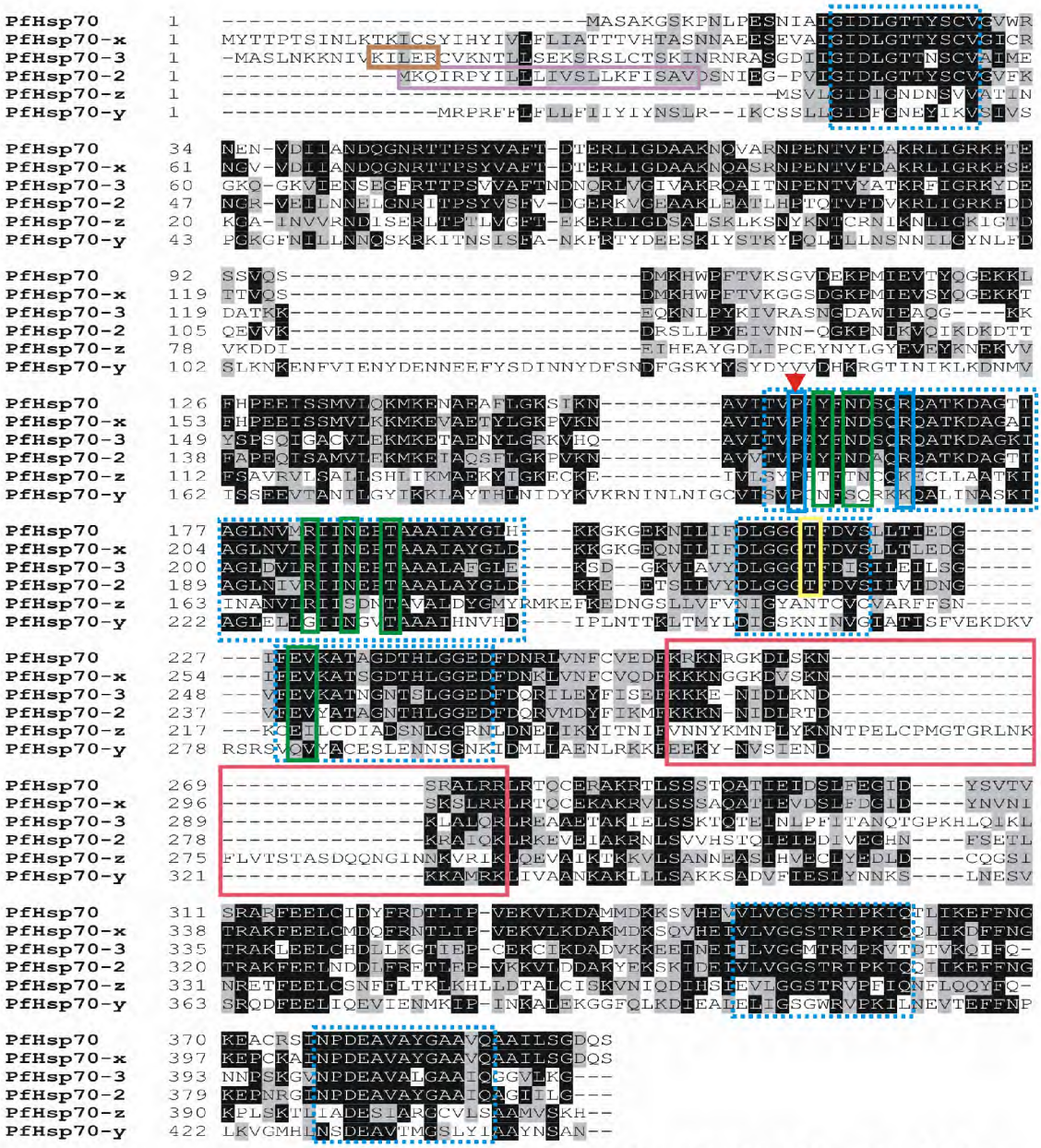

Figure 2.3 Sequence alignment for the ATPase domains of Hsp70 proteins from *Plasmodium falciparum*
Some of the key residues in the ATPase domain are highlighted as follows: host cell targeting signal (Lanzer *et al.*, 2006; brown box), ER N-terminal leader sequence (Munro and Pelham, 1986; maroon box), the bipartite nuclear localisation signal (Robbins *et al.*, 1991; red box) and highly conserved residues associated with heat shock proteins (Mount, 1985; blue boxes, with broken lines). Based on studies on DnaK, some residues that are important for its function and their corresponding residues in Hsc70 and PfHsp70 are highlighted as follows: P143, proline allosteric switch, marked by red arrow; and R151 both of which are important for interdomain function (Vogel *et al.*, 2006a; blue boxes with continuous line), Y145, N147, D148, N170 and T173 that interact with DnaJ (Gässler *et al.*, 1998; Suh *et al.*, 1998; green boxes) and T199, a DnaK phosphorylation site (McCarty and Walker, 1991; yellow box). Identical residues are presented in white in colour (on black background), whilst similar residues are shown in black colour (on grey background). The BioEdit programme (www.mbio.ncsu.edu/BioEdit/bioedit.html) ClustalW (Thompson *et al.*, 1994) based alignment option was used to carry out sequence alignment.

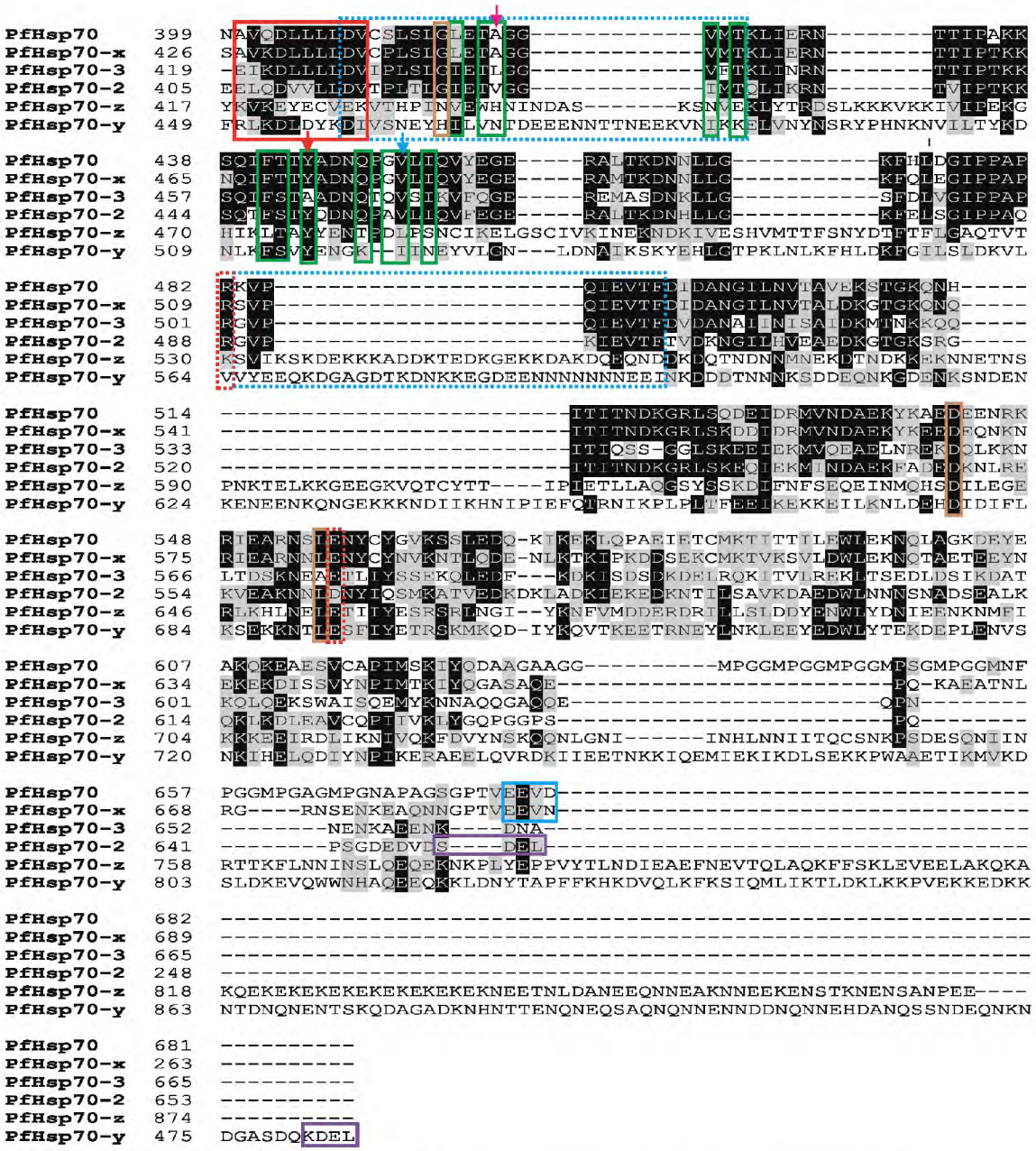

Figure 2.4 Sequence alignment of the peptide binding domains of Hsp70s from *Plasmodium falciparum*
Highlighted by the different rectangular boxes are the following residues: linker (red box); residues that are associated with heat shock proteins (Mount, 1985; blue boxes, with broken lines); terminal EEVD and EEVN motifs (blue box, with continuous lines). DnaK residues known to play key roles and corresponding residues in PfHsp70 and Hsc70 are also highlighted: residues that are in contact with the substrate (Zhu *et al.*, 1996; green boxes); and residues that interact with Hsp40 (Suh *et al.*, 1998; brown boxes). Residues that constitute the hydrophobic arch and hydrophobic pocket (Mayer *et al.*, 2000b) are shown (red arrows for arch residues and the blue arrow for hydrophobic pocket residue). The terminal Hsp70 ER retention signal (Kappes *et al.*, 1993; Pelham, 1989a,b; maroon boxes) is also shown as well as residues of *P. falciparum* Hsp70s that correspond to E543 and R469 of Hsc70 whose salt bridge stabilises the lid (Ha *et al.*, 1997; Chang *et al* 2001; red boxes, with broken lines). Identical residues are presented in white in colour (on black background), whilst similar residues are shown in black colour (on grey background). The BioEdit programme (www.mbio.ncsu.edu/BioEdit/bioedit.html) ClustalW (Thompson *et al.*, 1994) based alignment option was used to carry out sequence alignment.

2.3.2.2 Localisation signals on PfHsp70 and its *Plasmodium falciparum* homologues

PfHsp70 is mostly localised to the cytoplasm, but also occurs in the nucleus (Kappes *et al.*, 1993). It possesses the terminal EEVD motif present in eukaryotic cytoplasmic Hsp70s, whose role is to facilitate the interaction of Hsp70s with Hop and Hsp40 (Freeman *et al.*, 1995; Demand *et al.*, 1998). PfHsp70-x, which shares close homology and phylogenetic links with PfHsp70; has a terminal EEVN sequence (Figure 2.4), suggesting that it could also be a cytoplasmic chaperone (Sargeant *et al.*, 2006). Both PfHsp70 and PfHsp70-x have a highly conserved bipartite nuclear localisation signal (Figure 2.4; Robbins *et al.*, 1991). The nuclear localisation signal is also relatively conserved in PfHsp70-2 and PfHsp70-3. However, this signal sequence is weakly conserved in PfHsp70-y. PfHsp70-z has the least conserved residues for this signal compared to other *P. falciparum* Hsp70s. PfHsp70-3 contains a host cell targeting signal (Lanzer *et al.*, 2006), and PfHsp70-2 has an ER N-terminal leader sequence and a C-terminal ER-retention signal, both of which seem to confine it to ER-like structures in *P. falciparum* (Kumar *et al.*, 1991).

2.3.2.3 Phylogenetic analysis of PfHsp70 and its homologues from *Plasmodium falciparum*

Phylogenetic analysis was conducted on protein sequences of Hsp70s from *P. falciparum* in comparison with their homologues from other species (Figure 2.5). PfHsp70 and PfHsp70-x clustered with cytosolic Hsp70s of eukaryotic origin. The close phylogenetic orientation of PfHsp70-x to PfHsp70, coupled with the fact that it carries a terminal EEVN motif (Figure 2.4) that resembles the cytosolic marker sequence for eukaryotic Hsp70 (the terminal EEVD motif present in PfHsp70) strongly suggest that this protein is a second cytosolic Hsp70 for *P. falciparum*. PfHsp70-2 (PfGrp78) shows a close phylogenetic link to its human homologue, BiP (Grp78). The *P. falciparum* mitochondrial candidate, PfHsp70-3 forms a monophyletic clade with its *Trypanosoma cruzi* mitochondrial homologue (TcmtHsp70). Closely clustering with this group, are the prokaryotic cytosolic Hsp70s from *E. coli* (EcDnaK) and *Agrobacterium tumafaciens* DnaK (AtDnaK).

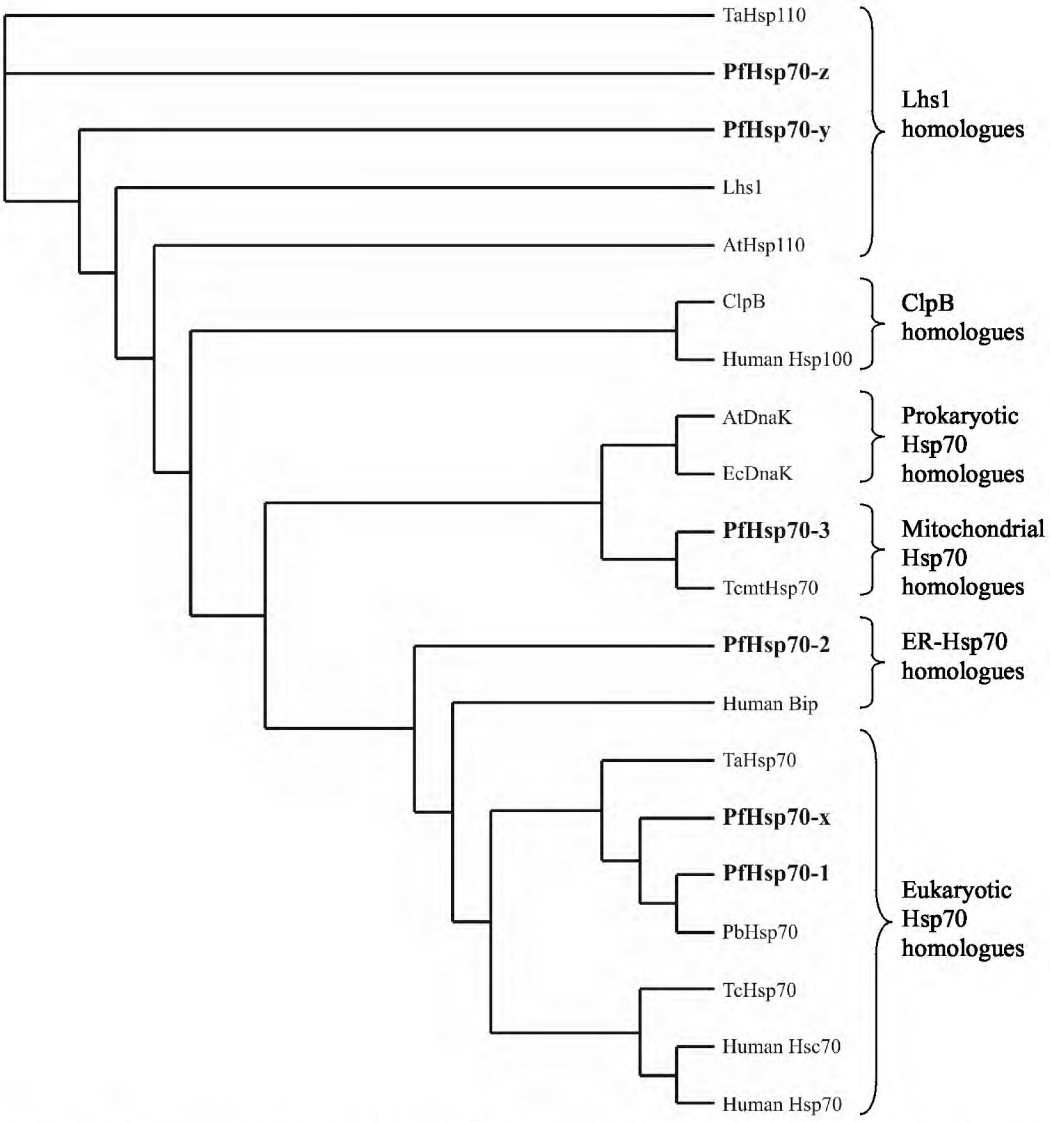

Figure 2.5 Phylogenetic analysis of *Plasmodium falciparum* Hsp70s and their homologues from other species

Hsp70-like proteins (bold letters) from *P. falciparum* and other sources were analysed. NCBI accession numbers of proteins from other organisms, besides *P. falciparum* are as follows: *Theileria annulata* Hsp110 (TaHsp110, XP_952474); *Arabidopsis thaliana* Hsp110 (AtHsp110, NP_567510); Yeast Lhs1 (Lhs1, P36016); *E. coli* ClpB (ClpB, AAB49540); human Hsp100 (NP_006651); *Agrobacterium tumafaciens* DnaK (AtDnaK, AAR84665); *E. coli* DnaK (EcDnaK, BAA01595.1); *Trpanosoma cruzi* mitochondrial Hsp70 (TcmtHsp70, AAA30215); human BiP (CAA61201); *Theileria annulata* Hsp70 (TaHsp70, A44985); *Plasmodium berghei* Hsp70 (PbHsp70, AAL34314); *Trypanosoma cruzi* Hsp70 (TcHsp70, P05456) and human Hsc70 (AF352832). The Hsp70-like proteins were subdivided into different subgroups as shown on the right hand side. Dendrograms were constructed using the BioEdit (www.mbio.ncsu.edu/BioEdit/bioedit.html)-Protdist neighbour phylogenetic analysis tool. The TreeView (Page, 1996) option housed on the BioEdit program was used to generate the dendrograms.

The close phylogenetic association of PfHsp70-y and PfHsp70-z with Hsp70-like proteins of the higher order molecular masses such as TaHsp110 from an apicomplexan species, *Theileria annulata* and AtHsp110 of plant origin (*Arabidopsis thaliana*), hints at the fact that

the two Hsp70-like proteins are Grp170 homologues. Grp170 proteins are Hsp70-like chaperones whose structural-functional features are distinct from Hsp70s (Easton *et al.,* 2000). PfHsp70-y carries a putative terminal ER-retention signal (KDEL). Lhs1 (Craven *et al.*, 1996), a yeast Hsp70-like protein that belongs to the Hsp110/Grp170 subfamily, shares close phylogenetic links with PfHsp70-y. Interestingly, Lhs1 has been established as a NEF for the ER-resident Hsp70, BiP (Steel *et al.*, 2004). Therefore, it is possible that PfHsp70-y is a *P. falciparum* ER-based Hsp70 NEF.

2.3.2.4 Conservation of PfHsp70 residues that interact with substrate

Residues corresponding to those of DnaK that occur in physical contact with the substrate (Zhu *et al.*, 1996) are highly conserved in the *P. falciparum* Hsp70 kingdom, although PfHsp70-y and PfHsp70-z show high divergence from the rest with respect to this aspect (Figure 2.4). Although there is general consensus in residues that physically interact with substrate for all the other four Hsp70s, there is significant variation in arch residues (Figure 2.4). Whereas the arch of *E. coli* DnaK's substrate binding cavity is made up of M404 and A429, in most eukaryotic Hsp70s, the residues corresponding to these are A and Y, respectively. Therefore, only PfHsp70 and PfHsp70-x have a typical eukaryotic Hsp70 arch architecture (Figure 2.4; Zhu *et al.*, 1996). In addition, only PfHsp70-y and PfHsp70-z do not possess the conserved valine residue that defines the hydrophobic pocket of the substrate binding cleft of most Hsp70s (Mayer *et al.*, 2000b).

A three-dimensional model of PfHsp70 showing the arch and hydrophobic pocket residues, together with all the other residues that have contact with substrate is shown (Figure 2.6). The acidic and hydrophobic peptide substrate that is favoured by Hsp70 (Rüdiger *et al.*, 1997) gets bound in the substrate binding cavity. As expected, PfHsp70 possesses a substrate binding cavity that is rich in hydrophobic residues; as is true for other Hsp70 proteins (Zhu *et al.*, 1996).

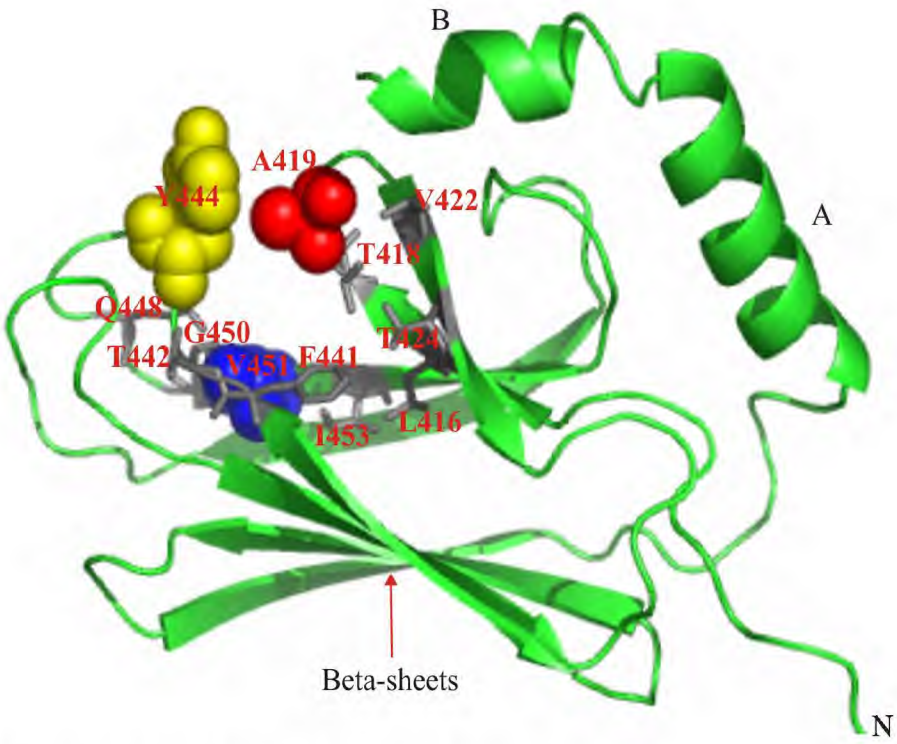

Figure 2.6 Arch and hydrophobic pocket residues of PfHsp70
A model of the β-sheet enriched substrate binding cavity of PfHsp70 showing residues that interact physically with substrate (shown as grey sticks). Of these, residues A419 (red spheres) and Y444 (yellow spheres) represent the arch. The hydrophobic pocket is represented by the residue V451 (blue spheres). Subdomains A and B of the C-terminal helical lid are also shown. 'N' represents the N-terminus. The model for the PBD of PfHsp70 was generated based on the crystal structure of bovine Hsc70 (1YUW.pdb; Jiang *et al.,* 2005). The figure was visualised using PyMol (DeLano, 2002).

2.3.2.5 Evidence for the potential regulation of PfHsp70 by Hsp40

Several Hsp40 binding sites were identified as conserved in the ATPase and peptide binding domains of *E. coli* DnaK, PfHsp70 and Hsc70 (Figures 2.1 and 2.2; Gässler *et al.*, 1998; Suh *et al.*, 1998). Using a three-dimensional model of PfHsp70 partial full length, the locations of its putative Hsp40 binding sites were illustrated (Figure 2.7; Gässler *et al.*, 1998; Suh *et al.*, 1998). In addition, the presence of residues D164 and E230 ensures that this cavity is negatively charged so that it could interact with the positively charged Hsp40 J domain. Of interest, is the fact that PfHsp70 residues (P159 and R167; Figure 2.7) corresponding to those implicated in the allosteric communication of DnaK (Vogel *et al.*, 2006a) cluster with Hsp40 binding sites in the predicted structure of PfHsp70 (Figure 2.7). Using a partial full length crystal structure of Hsc70, it was established that the Hsp40 binding cleft occurs close to the linker, in proximity with other residues important for its allosteric function (Jiang *et al.*, 2005).

Table 2.2 Localisation of PfHsp70 and its possible Hsp40 partners

Organelle	Type of Hsp70 present	Hsp40s present
P. falciparum cytoplasm	PfHsp70[a,j], PfHsp70-2 (PfGrp78)[j], PfHsp70-x[a]	At least 24 PfHsp40s[a], including the PfJ1[k] (type I), PF14_0359[a] (type I); PfJ4[k] (type II)
Parasitophorous vacuole	PfHsp70[b], PfHsp70-3[b]	None mentioned in this study[b]
Maurer's cleft	PfHsp70[c,d], PfHsp70-2[c,d], PfHsp70-3[c,d]	Resa-like Hsp40 (PFD1170c)[c,d]; PFE0055c (type II)[c,d]
Red blood cell cytoplasm	Host Hsp70[h], PfHsp70[g]-NDE	Export of at least 19 PfHsp40s-NDE[a,c,d,e,f]

Abbreviations for terms used in the table: NDE-No direct evidence. The following are the references for data used in constructing the table: a-Sargeant *et al.*, 2006; b-Nyalwidhe and Lingelbach, 2006; c-Lanzer *et al.*, 2006; d-Vincensini *et al.*, 2005; e-Hiller *et al.*, 2004; and f-Templeton and Deitsch, 2005; and g-Kumar *et al.*, 1990, h-Banumathy *et al.*, 2002; j-Kumar *et al.*, 1991; k-Watanabe, 1987

PfHsp70 occurs in the cytoplasm and the nucleus (Table 2.2; Kumar *et al.*, 1991). At least 43 Hsp40-like proteins have been identified on the *P. falciparum* genome (Sargeant *et al.*, 2006). Of these nineteen are predicted to be exported based on export signal motifs that they carry (Templeton and Deitsch, 2005; Sargeant *et al.*, 2006). Therefore, there are 24 Hsp40 proteins that could be restricted to the cytoplasm of *P. falciparum* (Table 2.2). Amongst these, two type I Hsp40s (PfJ1 and PF14_0359 Table 2.2; Watanabe, 1997; Sargeant *et al.*, 2006) are predictably confined to *P. falciparum* cytoplasm (Sargeant *et al.*, 2006). It is possible that at least one of these two type I Hsp40s could be PfHsp70's co-chaperone partner. However, PfHsp70-2 is also found in the cytoplasm (Kumar *et al.*, 1991). PfHsp70-x is also thought to occur in the cytoplasm of *P. falciparum* (Table 2.2; Sargeant *et al.*, 2006; this study). Therefore, the possible Hsp40 that could match each *P. falciparum* Hsp70 are not obvious based on localisation data. However, a type II Hsp40, PfJ4, whose mRNA is heat-inducible has been partially characterised (Watanabe, 1997). The fact that PfJ4 is possibly heat-induced makes it a candidate Hsp40 partner for PfHsp70 since the latter is also heat-induced (Kumar *et al.*, 1991)

Assuming that PfHsp70 is exported to the red blood cell, it would be expected that its Hsp40 partners could be exported as well so that they could regulate PfHsp70 in all the oragnelles where it is found. The potential export of PFE0055c (a type II Hsp40) to the Maurer's cleft (Table 2.2; Vincensini *et al.*, 2005; Lanzer *et al.*, 2006) along with the *P. falciparum* Hsp70s (PfHsp70, PfHsp70-2 and PfHsp70-3) suggests a role for this Hsp40 in the regulation of *P. falciparum* Hsp70 proteins.

2.3.2.6 PfHsp70 possesses loops and salt bridges important for nucleotide exchange

A conserved loop region that occurs in subdomain IB of DnaK is thought to ensure the stable interaction of DnaK with GrpE (Figure 2.8; Buchberger *et al.*, 1994). This loop is also implicated in the regulation of nucleotide binding by DnaK and its role is essential for DnaK function (Buchberger *et al.*, 1994). In addition, a salt bridge that is formed by R34 and E369 in DnaK which is conserved in other Hsp70s regulates nucleotide exchange by preventing nucleotide release in the absence of GrpE, and opens up to release nucleotide when GrpE binds to the neighbouring loop (Figure 2.8A; Buchberger *et al.*, 1994). Salt bridges that are equivalent to the R34-E369 salt bridge of DnaK (Buchberger *et al.*, 1994) are represented in both Hsc70 and PfHsp70 (Figure 2.8). In addition, DnaK has two extra salt bridges that link subdomain IB and IIB that further regulate nucleotide exchange (Brehmer *et al.*, 2001). On the other hand, both PfHsp70 and Hsc70 only have one such salt bridge each (Figure 2.8).

Another important structural motif for interaction of DnaK with GrpE, the GrpE signature loop, is located in subdomain IIB of the ATPase domain (Figure 2.8A; Brehmer *et al.*, 2001). Within the GrpE signature motif is a substructure, the GrpE motif (Figure 2.8A; Brehmer *et al.*, 2001). The GrpE motif in DnaK forms a loop that is essential for its interaction with GrpE (Figure 2.8A and 2.8B; Brehmer *et al.*, 2001). Both PfHsp70 and Hsc70 have a GrpE motif that is structurally divergent from that of DnaK (Figure 2.8). The fact that PfHsp70 has high sequence similarity to human Hsc70 in the critical GrpE motif section (Figures 2.8A and 2.8B; Brehmer *et al.*, 2001) suggests that NEFs that interact with Hsc70 could also recognise PfHsp70.

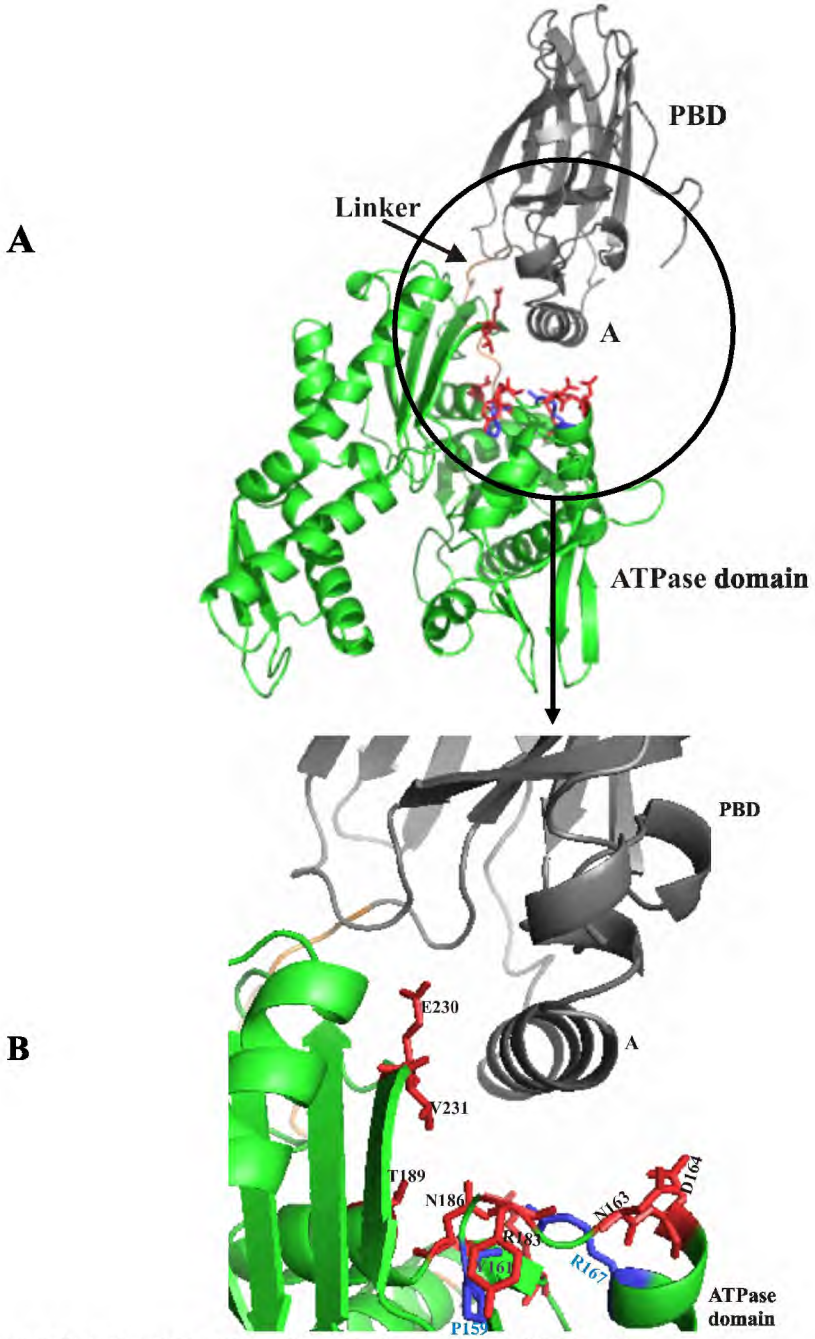

Figure 2.7 Putative PfHsp70 residues for interaction with Hsp40 partners
(**A**) Distribution of some highly conserved Hsp40 binding sites (depicted as red sticks) in the ATPase domain of PfHsp70 (Gässler *et al.*, 1998; Suh *et al.*, 1998). (**B**) A zoomed representation of the Hsp40 binding cleft encircled in A, showing different residues of PfHsp70 implicated in implicated in interdomain communication and interaction with Hsp40. Included in the picture, is putative PfHsp70 proline switch (P159) and residue R167 (depicted as blue sticks, with blue labels) that are equivalent to P143 and R151 important for allosteric interaction of DnaK (Vogel *et al.*, 2006a). The linker that connects the peptide binding domain (PBD) and ATPase domain is shown in orange colour. The α-helical subdomain A that is situated in the proximal environment of the Hsp40 binding site is shown. The three dimensional structure of PfHsp70 was generated by modelling against the crystal structure of bovine Hsc70 (1YUW.pdb; Jiang *et al.*, 2005). The image was visualised using PyMol (DeLano, 2002).

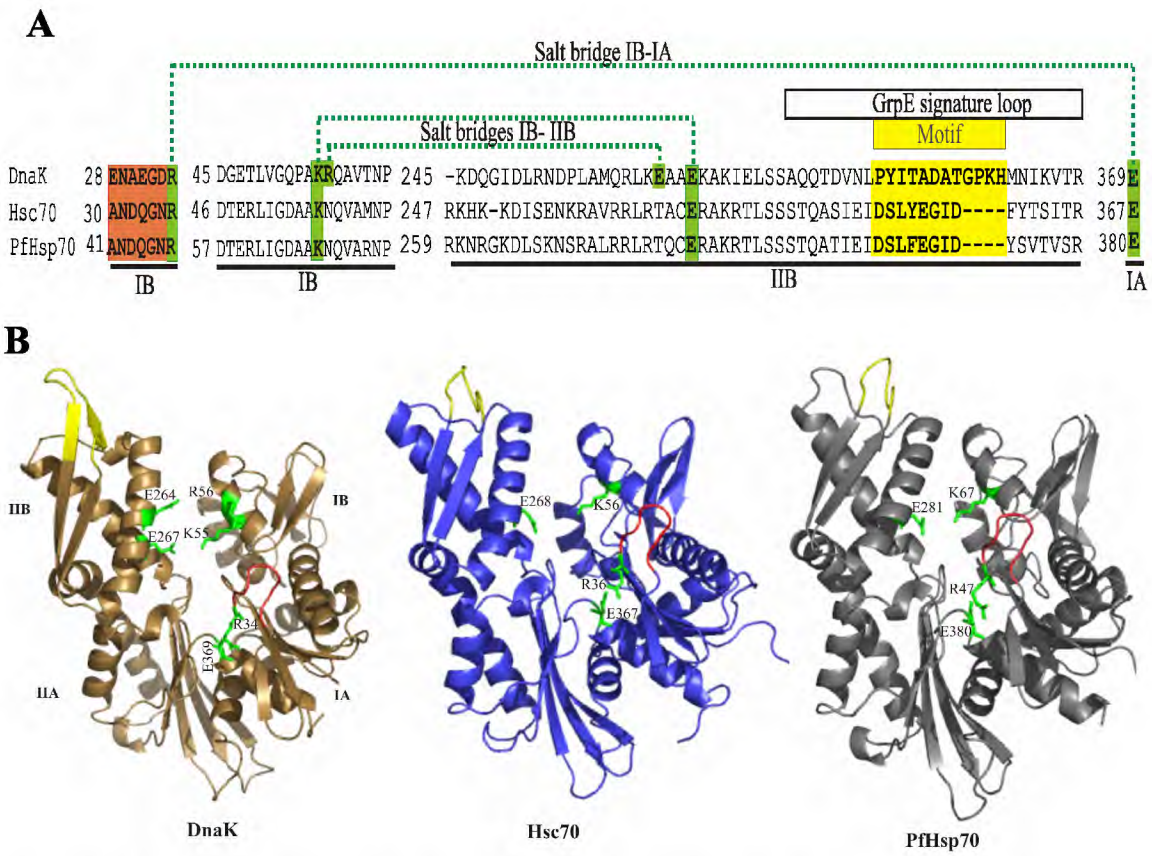

Figure 2.8 Conservation of Hsp70 interdomain loops and salt bridges involved in nucleotide exchange
Conservation of residues constituting interdomain loops and salt bridges in DnaK, Hsc70 and PfHsp70 (**A**) and three-dimensional images for the ATPase domains of DnaK, Hsc70 and PfHsp70 (**B**) showing the loops and salts crucial in nucleotide exchange. Residues in subdomain IB (red) form a highly conserved loop and salt bridge (green) that links subdomains IA and IB. The loop and salt bridge are both important for nucleotide exchange in DnaK (Buchberger *et al.*, 1994). DnaK has two additional salt bridges that link subdomain IB and IIB (green). Of these Hsc70 and PfHsp70 each has only one. Another additional feature that confers specificity to the interaction of NEF with a particular Hsp70 is the GrpE signature loop whose tip (motif, highlighted in yellow) is made up of highly divergent residues in Hsp70s (Brehmer *et al.*, 2001). The three dimensional images for the ATPase domains of human Hsc70 and PfHsp70 were generated by modelling them against the bovine Hsc70 (1YUW.pdb; Jiang *et al.*, 2005). The images of *E. coli* DnaK (1DKG.pdb), human Hsc70 and PfHsp70 were visualised using PyMol (DeLano, 2002). The figure was adapted from Brehmer *et al.* (2001).

PfHsp70 and Hsc70 share high sequence similarity in residues that were identified as key Bag-1 and HspBP1 contact points (Figure 2.9; Sondermann *et al.*, 2001; Shomura *et al.*, 2005). Subdomain IIB houses most of these residues, and of particular importance is that the five residues that are used for both Bag-1 and HspBP1 contact also occur in this subdomain (Figure 2.9). Bag-1 and HspBP1 contact residues are highly conserved in PfHsp70 and most of them are solvent exposed, based on the predicted three-dimensional structure of PfHsp70 (Figure 2.9; Sondermann *et al.*, 2001; Shomura *et al.*, 2005). Residues that are solvent exposed are potential candidates for protein-protein interaction (Rajamani *et al.*, 2004).

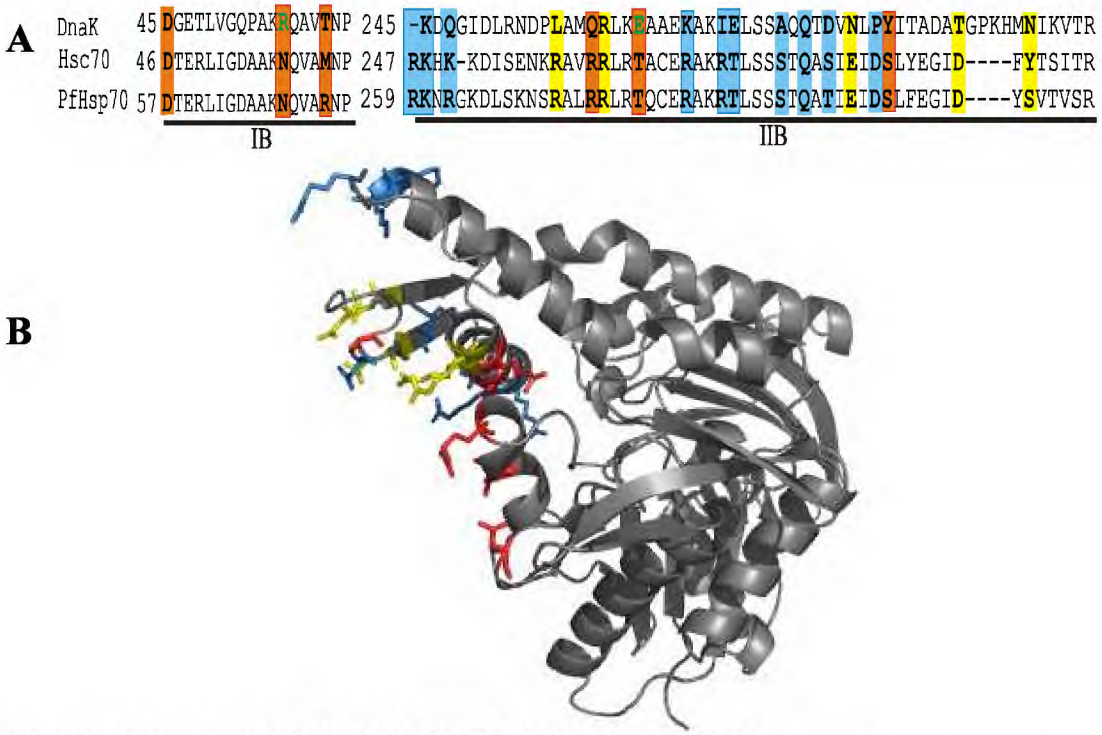

Figure 2.9 Conservation of Bag-1 and HspBP1 contact sites in PfHsp70
(**A**) Residues of Hsc70 that interact with HspBP1 and Bag-1 (Shomura *et al.*, 2005; Sondermann *et al.*, 2001) together with their corresponding residues in PfHsp70 and DnaK are shown. Bag-1 contact sites are (red) and HspBP1 contact sites (blue) are highlighted. Residues that are implicated as both Bag-1 and HspBP1 contact sites are shown in yellow background. (**B**) Plan view of the three dimensional structure of PfHsp70 ATPase domain showing the exposure to solvent of residues for contact with Bag-1 (red), HspBP1 (blue) and contact residues common to both NEFs (yellow). The ribbon structure of the ATPase domain of PfHsp70 was generated through modelling against the crystal structure of bovine Hsc70 ATPase domain (1YUW.pdb). The image was visualised using PyMol (DeLano, 2002).

2.4 The potential interactome of *Plasmodium falciparum* Hsp70 proteins

Based on yeast-two hybrid analysis, several *P. falciparum* proteins that potentially interact with Hsp70s from this parasite were identified (Figure 2.10; LaCount *et al.*, 2005). Three major groups of proteins (cytoskeletal and membrane protein; proteins involved in DNA repair and replication; and proteins and enzymes involved in cell physiology and metabolism) accounted for approximately one third each of the total proteins potentially involved in *P. falciparum* Hsp70 network (Figure 2.10). Using the concept of 'bait' and 'prey'; most of the associations between a particular Hsp70 and its partner protein involved the chaperone acting as the 'bait'. Reciprocal association of 'bait' and 'prey' which strongly confirms the interactions of the proteins in the network were not observed for any of the Hsp70s and their potential partner proteins recorded here (Figure 2.10).

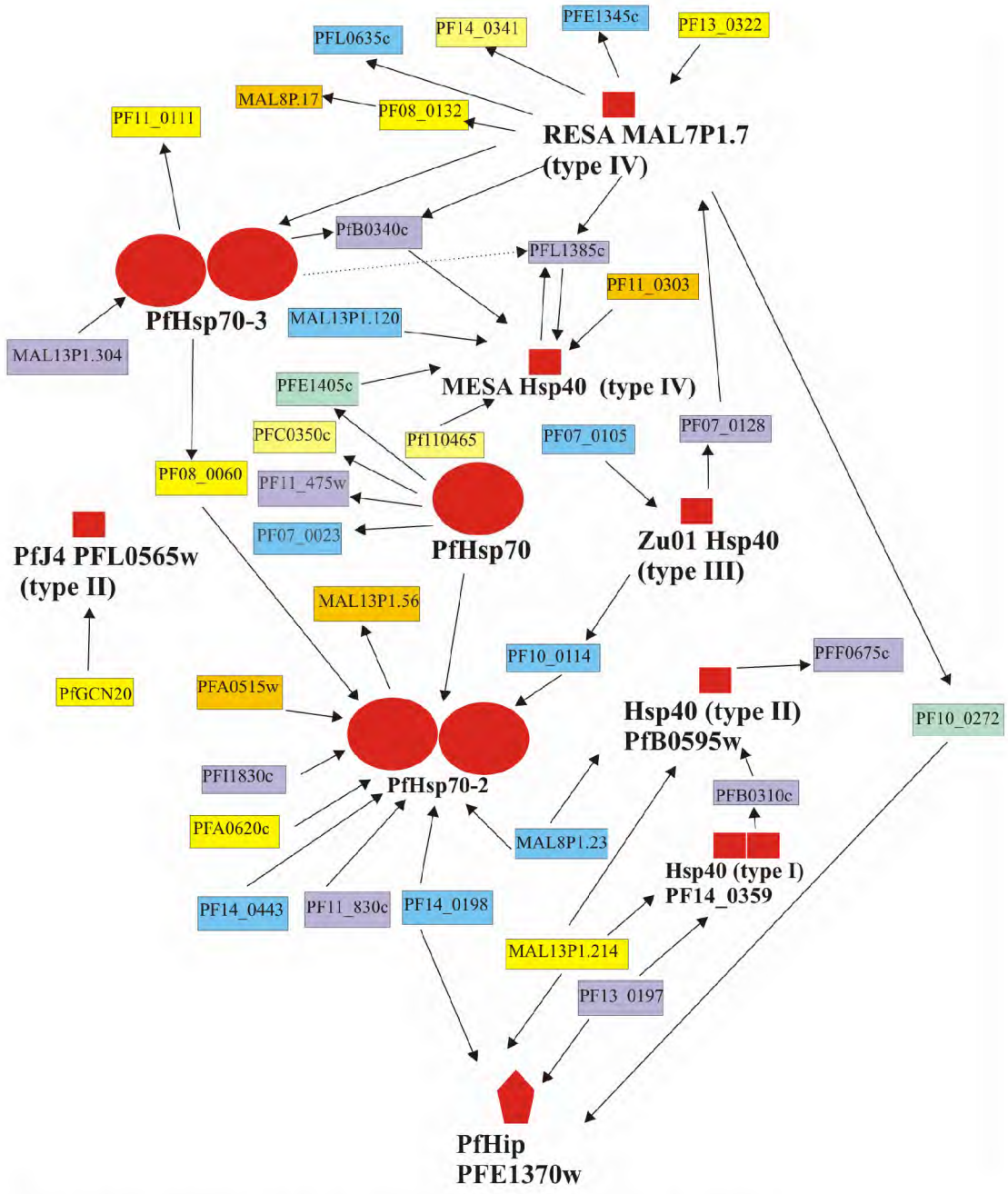

Figure 2.10 The potential *Plasmodium falciparum* Hsp70 interactome of proteins
The network of interactions involving Hsp70s from *P. falciparum* and other was constructed based on data from yeast-two hybrid studies (LaCount *et al.*, 2005). The chaperones are presented as follows: Hsp70s (red spheres), Hsp40-like proteins (red rectangles) and PfHip (red pentagon). The rest of the proteins involved in the network are classified as: cytoskeletal and membrane proteins (purple), translational and transcriptional machinery (green), proteosome and proteolytic enzymes (orange), enzymes involved in physiological and metabolic pathways (yellow) and DNA repair and replication enzymes (blue). The arrow points in the direction of the prey. Reciprocal association is represented by arrows facing opposite directions. A broken arrow links PfHsp70-3 and PFL1385c. Self association of chaperones is illustrated by two adjacent shapes representing the particular protein.

Self association was observed for the following chaperones: PfHsp70-2, PfHsp70-3 and a type I Hsp40 protein (PF14_0359). Strangely, there is potential interaction observed between PfHsp70 and PfHsp70-2. It is not clear whether this association does really occur practically in *P. falciparum*. PfHsp70-2 had the largest pool of protein network partners identified in this study, followed by PfHsp70 and PfHsp70-3 in descending order. The only interaction between an Hsp70 and an Hsp40-like protein was that involving RESA-like Hsp40 and PfHsp70-3. It is conceivable that the limitations of the technique employed by LaCount *et al.* (2005) may have led to their failure to detect transient protein-protein interactions such as those between Hsp70 and the Hsp40 partner (Pierpaoli *et al.*, 1998). However, there are several proteins that were identified as potential common interaction network partners for Hsp70s and some Hsp40-like proteins. For example, a putative ubiquitin protein ligase 1 (MAL8P1.23) was shown to interact with both PfHsp70-2 and a type II Hsp40-like protein (PfB0595w). In addition, PfHsp70-2 associated with a putative DNA repair protein (PF10_0114) which in turn interacted with Zuo1 type III Hsp40-like protein. In this study, the term type IV Hsp40-like proteins has been used to describe Hsp40-like proteins with only the J domain as their Hsp40 marker feature, but lack a conserved HPD motif. Some type IV Hsp40-like proteins; RESA- and mature parasite-infected erythrocyte surface antigen (MESA)-like Hsp40 subgroups interacted with some proteins that in turn associated with Hsp70 proteins. Another co-chaperone, PfHip (PFE1370w) interacted with a putative tRNA glycine ligase (PF14_0198) which in turn associated with PfHsp70-2. There is evidence that PfHip is able to modulate the chaperone function of *P. falciparum* Hsp70s and its interaction with PfHsp70 and PfHsp70-3 has been observed *in vitro* (Ramya *et al.*, 2006).

2.5 Discussion

Using *E. coli* DnaK as a model, PfHsp70 residues potentially important for its interaction with its Hsp40 partner(s) (Gässler *et al.*, 1998; Suh *et al.*, 1998) were identified (Figures 2.1 and 2.2). Interestingly, most of these residues are conserved in both the ATPase and peptide binding domains of PfHsp70; consistent with the fact that Hsp70s interact with their Hsp40 partners in a bipartite fashion (Figures 2.1 and 2.2; Suh *et al.*, 1998). The residues are not only highly conserved in PfHsp70 (Figure 2.1), but they are also predictably surface-exposed (Figure 2.7B); and this is congruent with the organisation of similar residues in DnaK (Gässler *et al.*, 1998).

Since PfHsp70 occurs in the cytoplasm (Kumar *et al.*, 1991), its Hsp40 co-chaperones would be expected to be found in this structure. For example, there are two type I Hsp40s in *P. falciparum*: PfJ1 (Watanabe, 1997) and PF14_0359 that are predictably not exported (Table 2.2; Sargeant *et al.*, 2006). PF14_0359 has 424 amino acids compared to PfJ1 which is 672 amino acids long. Based on size, PfJ1 seems too big to be a typical Hsp40 protein. Therefore, PF14_0359 would appear to be PfHsp70's cytoplasmic type I Hsp40 partner. It is possible that one of the parasite-resident type II Hsp40s could be PfHsp70's co-chaperone partner. For example, PfJ4 (type II Hsp40 protein) (Table 2.2), is a potential Hsp40 partner of PfHsp70 since its mRNA is heat inducible, hence it is speculated that its production could be stress-invoked (Watanabe, 1997). Since PfHsp70 is induced in response to heat stress (Kumar *et al.*, 1991), it would be practically beneficial that its Hsp40 co-chaperone be heat-induced as well.

Of the 43 *P. falciparum* Hsp40-like proteins, at least nineteen Hsp40 proteins are predicted to be exported based on export signal motifs that they carry (Templeton and Deitsch, 2005; Sargeant *et al.*, 2006). Assuming that the three Hsp70s that have been reported beyond the parasite cytoplasm (Table 2.2; Vincensini *et al.*, 2005; Lanzer *et al.*, 2006; Nyalwidhe and Lingelbach, 2006) are exported to the erythrocyte, it is reasonable that nineteen Hsp40-like proteins could be shuttled beyond the cytoplasm of *P. falciparum* (Templeton and Deitsch, 2005; Sargeant *et al.*, 2006) to accompany their respective Hsp70 partners. This is possible given the fact that several Hsp40s can serve one Hsp70 partner (Cyr, 1995; Walsh *et al.*, 2004; Hennessy *et al.*, 2005). However, it has been hypothesised that this large contingent of Hsp40-like proteins could be exported in order to modulate the function of their *P. falciparum* Hsp70 partners, as well as interact with host Hsp70s in the red blood cell (Sargeant *et al.*, 2006). This is persuasive, since host Hsp70s are known to associate with

some proteins exported from *P. falciparum* (Banumathy *et al.*, 2002). Although, no direct evidence confirms that PfHsp70 is exported to the red blood cell, the detection of antibodies to PfHsp70 in malaria patients suggests that this protein could join the host circulatory system, thereby invoking an immune response (Kumar *et al.*, 1990). Because PFE0055c (a type II Hsp40) has been proposed to be present in the Maurer's cleft (Table 2.2; Vincensini *et al.*, 2005; Lanzer *et al.*, 2006), together with PfHsp70, there is reason to speculate that it could be PfHsp70's co-chaperone partner. Only type I and type II Hsp40s are able to modulate the ATPase activity of their Hsp70 partners as well as interact with substrate (Walsh *et al.*, 2004). It is possible however, that PFE0055c could also be the Hsp40 co-chaperone of PfHsp70-2 or PfHsp70-3, both of which have been reported to be in the Maurer's cleft together with PfHsp70 (Table 2.2; Vincensini *et al.*, 2005; Lanzer *et al.*, 2006). However, the proposed localisations of *P. falciparum* proteins as observed by Vincensini *et al.* (2005), though a useful guide of the possible *P. falciparum* exportome, requires further experimental evidence to unequivocally confirm the localisation of these proteins.

Data based on the Hsp70 interactome (Figure 2.10; LaCount *et al.*, 2005), could be useful for inferring potential Hsp40-Hsp70 partnerships. For example, the fact that the putative ubiquitin protein ligase 1 (MAL8P1.23) interacted with both PfHsp70-2 and a type II Hsp40-like protein (PfB0595w) suggests that PfB0595w could be the Hsp40 partner of PfHsp70-2. However, most other such associations link type III binding to proteins that also interacted with Hsp70s (Figure 2.10). Although it is logical to infer that an Hsp40-like protein that has a common ligand to a particular Hsp70 could be involved in a functional partnership with that particular Hsp70, this is highly unlikely to be the case when a type III Hsp40 is involved. This is because the mechanism by which type III Hsp40s interact with Hsp70s is not well defined, although there is evidence that some type III Hsp40 proteins co-operate with Hsp70 proteins (Ungewickell *et al.*, 1995; Gall *et al.*, 2000; Xiao *et al.*, 2006). Some type IV Hsp40-like proteins associated with some proteins that also interacted with Hsp70s (Figure 2.10). Whether such association suggests a possible partnership for such Hsp40-like proteins and the respective Hsp70s is very obscure since type IV Hsp40-like proteins do not have the conserved HPD motif that is essential for the interaction of Hsp40s with Hsp70s (Mayer *et al.*, 1999).

Allosteric communication is an essential feature of Hsp70 protein function, regulating both substrate release and ATP hydrolysis (Han and Christen, 2003). The elucidation of the partial full-length structure of bovine Hsc70 by Jiang *et al.* (2005) revealed structural details that suggest that the interaction between Hsp70 and Hsp40 occurs at the linker interface, allowing Hsp40 to interact with both the ATPase and peptide binding domain of Hsp70. Not surprisingly, some residues on Hsp70 that are important for interaction with Hsp40 are implicated in interdomain communication (Gässler *et al.*, 1998; Jiang *et al.*, 2005). In support of this, the predicted structure of PfHsp70 displays an Hsp40 binding cleft in which residues perceived to be important for interaction with Hsp40 are closely associated with those essential for interdomain communication, including the equivalence of the DnaK proline allosteric switch (Figure 2.7; Vogel *et al.*, 2006a). The fact that both PfHsp70-y and PfHsp70-z, lack a distinct linker structure that is crucial for allosteric control of Hsp70s (Han and Christen, 2001, Vogel *et al.*, 2006b) suggests that these Hsp70s are differently regulated or have a distinct role that is divergent from the typical Hsp70 chaperone function. For example, the fact that PfHsp70-y contains a putative terminal ER-retention signal and shows close phylogenetic connection to the yeast Hsp70-like Hsp110/Grp170 homologue, Lhs1 (Steel *et al.*, 2004), suggests that PfHsp70-y could be a NEF for PfHsp70-2. Besides Lhs1 (Steel *et al.*, 2004), another yeast Hsp110, SseIp has been identified as the role of a NEF (Dragovic *et al.*, 2006). Therefore, it is possible that both PfHsp70-z and PfHsp70-y could act as NEFs of the Hsp110/Grp170 family.

Hsp70s are classified as DnaK, HscA or Hsc70 protein families, according to their mechanism of interaction with NEFs (Brehmer *et al.*, 2001). Key to this classification system, are the salt bridges between subdomains IB and IIB and a segment that is referred to as the GrpE signature loop (Figure 2.8; Brehmer *et al.*, 2001). Based on this decisive feature, there is evidence that PfHsp70 falls into the category of cytosolic Hsp70s (Figure 2.8; Brehmer *et al.*, 2001; Sondermann *et al.*, 2001). Insight from the organisation of salt bridges implicated in nucleotide exchange, suggests that PfHsp70 is more structurally inclined to human Hsc70 than *E. coli* DnaK (Figure 2.8). Whereas DnaK has two salt bridges linking subdomain IB to subdomain IIB, PfHsp70 and Hsc70 have only one each. However, the loop in subdomain IB and the accompanying salt bridge linking subdomain IA and IB of the ATPase domain is conserved in all the three Hsp70s (Figure 2.8). Not surprisingly, this loop is essential for DnaK nucleotide exchange (Buchberger *et al.*, 1994).

Despite their structural divergence, Bag-1 and GrpE interact with their Hsp70 partners through subdomain IB and IIB (Figure 2.9). However, the specific mechanistic details involving Bag-1 and GrpE interaction with their Hsp70 partners are not the same. GrpE functions as a dimer (Harrison *et al.*, 1997), as opposed to Bag-1 which uses a single domain (Sondermann *et al.*, 2001). GrpE has more contact points with DnaK than exist between Bag-1 and Hsc70 (Sondermann *et al.*, 2001; Chesnokova *et al.*, 2003). As expected, the two NEFs manifest different effects on their client Hsp70s. For example, whereas GrpE triggers the release of both ADP and ATP from DnaK, Bag-1 induces only ADP release (Brehmer *et al.*, 2001). HspBP1, though a eukaryotic NEF, binds and induces nucleotide exchange conformation in Hsp70 proteins in a different mechanism from Bag-1 (Shomura *et al.*, 2005).

An investigation of the nature of potential NEF binding sites on PfHsp70 was carried out in order to gain insight into the nucleotide exchange system of this chaperone (Figures 2.8 and 2.9). It is interesting to note that of the five Hsc70 residues that interact with both Bag-1 and HspBP1, PfHsp70 has four of these residues identical to corresponding residues in Hsc70. Of particular note is the observation that most of the residues implicated in Bag-1 and HspBP1 contact (Shomura *et al.*, 2005; Sondermann *et al.*, 2001) are not only conserved in PfHsp70, but they are also predicted to be solvent exposed (Figure 2.9B) suggesting their accessibility for protein-protein interaction (Rajamani *et al.*, 2004). It is envisaged that nucleotide exchange in PfHsp70 could occur through the same mechanism as that of Hsc70, since most of the residues of PfHsp70 implicated in its interaction with NEF are similar to those of Hsc70. *P. falciparum* contains a GrpE homologue (PF11_0258). However, GrpE homologues of eukaryotic origin such as yeast are located in the mitochondria (Westermann *et al.*, 1995). Therefore, since PfHsp70 is cytosolic, PfGrpE may not be its NEF.

Phosphorylation of Hsp70 proteins is an important functional regulatory aspect of their chaperone role (McCarty and Walker, 1991; Sherman and Goldberg, 1993). It ensures that Hsp70 proteins act as thermosensors that can easily step up, not only their levels during physiological stress, but improve the capacity of their chaperone function as well (McCarty and Walker, 1991). Therefore, the fact that at least four of the *P. falciparum* Hsp70s possess a threonine residue that corresponds to the DnaK phosphorylation site (T199; Figure 2.3, McCarty and Walker, 1991) suggests that most of them may have phosphorylation regulated activities. Indeed, PfHsp70 and PfHsp70-2 display growth phase dependent phosphorylation *in vivo* through their threonine and serine residues (Kumar *et al.*, 1991; Kappes *et al.*, 1993).

Because there is greater sequence variation in the peptide binding domain than in the ATPase domain, it is believed that the peptide binding domain confers substrate specificity to Hsp70 proteins (Fourie *et al.*, 1994). However, the β-sandwich located in the peptide binding domain of Hsp70s that accommodates the residues that are involved in physical contact with the substrate is relatively conserved across Hsp70 species (Zhu *et al.*, 1996; Wu and Wang, 1999). Hsp70 proteins are known to prefer substrates that are rich in hydrophobic residues (Flynn *et al.*, 1991; Gragerov *et al.*, 1994). There is evidence that the physical attributes of the peptide binding domain of an Hsp70 protein is not the only determinant of its functional specificity since co-chaperones also regulate Hsp70 functional specificity (James *et al.*, 1997). PfHsp70 has a substrate binding cavity that is very similar to that of DnaK and Hsc70 since all three proteins display a high level of sequence conservation in this segment (Figure 2.2). However, the arch residues of PfHsp70 differ from those of *E. coli* DnaK. PfHsp70 possesses a typical eukaryotic arch in which the residues are inverted as compared to those in DnaK (Figure 2.6; Zhu *et al.*, 1996). However, its hydrophobic pocket residue as represented by the V451 residue is the same as that of *E. coli* DnaK (V436) and this residue is highly conserved in Hsp70s (Mayer *et al.*, 2000b). PfHsp70-x is another *P. falciparum* Hsp70 homologue that shares the same arch and hydrophobic pocket features as PfHsp70 (Figure 2.4). The lack of consensus in arch residues observed in other *P. falciparum* Hsp70s is consistent with the fact that the arch residues are the least conserved substrate binding residues, thus perfectly placing them as determinants of Hsp70 substrate specificity (Rüdiger *et al.*, 1997; Mayer *et al.*, 2000b).

The C-terminal helical lid is divergent across Hsp70s and because of this, its role in regulating Hsp70 substrate binding specificity has been proposed, although there is controversy regarding this (Pellecchia *et al.*, 2000; Moro *et al.*, 2004). However, the lid has been implicated in interdomain communication because of its proximity to the linker and the Hsp40 binding cleft (Figure 2.7; Jiang *et al.*, 2005). It has been proposed that when Hsp40 interacts with Hsp70, it perturbs the neighbouring α-helical A subunit; thus influencing peptide binding kinetics by Hsp70 (Jiang *et al.*, 2005). PfHsp70 displays a lid segment that resembles that of Hsc70 (Figure 2.2; Chou *et al.*, 2003) and has conserved residues corresponding to E543 and R469 known to be important for stabilising the lid of Hsc70 (Figure 2.2; Ha *et al.*, 1997; Chang *et al.*, 2001). This suggests that the same residues could be responsible for stabilising PfHsp70's lid, highlighting the possible role of this structure in the chaperone function of PfHsp70.

PfHsp70 has high identity to some, but not all the other five Hsp70s from *P. falciparum* (Table 2.1). It has the closest identity to PfHsp70-x (73%), followed by PfHsp70-2 (PfGrp78) with which it shares 53% (Table 2.1). The divergence of *P. falciparum* Hsp70s is well illustrated by data from the phylogenetic analysis (Figure 2.5) that illustrates the distribution of these Hsp70s across various Hsp70 phylogenetic clades. This structural divergence is expected since these proteins are localised in different cells organelles. PfHsp70 occurs in the cytoplasm and nucleus (Kappes *et al.*, 1993). The nuclear localisation of Hsp70 has implicated this group of proteins in the regulation of the cell cycle (Hunt *et al.*, 1999). Whether PfHsp70 plays this role remains to be confirmed. The Grp78 homologue (PfHsp70-2) is localised in the cytoplasm in ER-like structures (Kumar *et al.*, 1991). Incidentally, this protein has a putative malarial C-terminal ER-retention signal (SDEL; Figure 2.3) as compared to the KDEL motif that is standard in other Grp78 proteins (Pelham, 1989a). In addition, PfHsp70-2 contains an N-terminal ER leader sequence (Figure 2.4; Kappes *et al.*, 1993). Despite having motifs that suggest ER-retention, coupled to evidence from a study that confirmed its localisation in ER-like structures in the cytoplasm of *P. falciparum* (Kumar *et al.*, 1991), PfHsp70-2 does seem to translocate at least beyond the parasite cytoplasm and has been detected in the Maurer' cleft (Vincensini *et al.*, 2005). The fact that *P. falciparum* Hsp70s with host cell retention signals have been proposed to be channelled beyond the parasite cytoplasm is confounding (Table 2.2). This confirms the complexity involved in *P. falciparum* protein trafficking. However, extensive validation is needed to ascertain data on the localisation of *P. falciparum* proteins in light of these discrepancies.

This study established that PfHsp70 shares several structural and functional features with its prokaryotic and eukaryotic counterparts. However, there is need to carry out further experimental work in order to validate inferences on the chaperone features of PfHsp70 gained in this study. Chapter 3 is a report on work that was conducted to investigate the *in vivo* chaperone properties of PfHsp70 using an *E. coli* heterologous system.

Chapter 3

Characterisation of the chaperone properties of PfHsp70 using an *in vivo* system

3.1 Introduction

The thermo-inducible production of PfHsp70 in *P. falciparum* has been confirmed (Kumar *et al.*, 1991; Joshi *et al.*, 1992; Biswas and Sharma, 1994). The observed association between PfHsp70 and PfHsp90 in ATP-dependent association during immunoprecipitation studies suggest a possible *in vivo* functional partnership between these two chaperones (Banumathy *et al.*, 2003). There is growing evidence from *in vitro* studies showing that PfHsp70 has chaperone properties (Matambo *et al.*, 2004; Ramya *et al.*, 2006). However, the *in vivo* role of PfHsp70 in the life-cycle of *P. falciparum* remains largely unknown.

Using an *E. coli* heterologous complementation assay (Longshaw *et al.*, 2006), this study investigated PfHsp70's *in vivo* functional capabilities. *E. coli* DnaK is dispensable at intermediate temperatures (around 30°C) but becomes essential for growth at extreme temperatures (Bukau and Walker, 1989b). Even at the intermediate temperatures, *E. coli* cells devoid of DnaK display cell division defects and retarded growth (Bukau and Walker, 1989b). *E. coli* cells with a deleted DnaK, or whose DnaK is functionally compromised can be used as host cells for the recombinant expression of Hsp70s from other species to test the ability of the exogenously expressed proteins to functionally replace (complement) DnaK function (Longshaw *et al.*, 2006). The ability of the exogenously expressed Hsp70 proteins to reverse thermosensitivity in these *E. coli* strains confirms them as potential molecular chaperones.

A number of Hsp70 proteins from prokaryotic organisms were able to reverse the thermosensitivity of *E. coli dnaK* deficient cells when exogenously expressed in these cells (Cellier *et al.*, 1992; Michel, 1993; Eom *et al.*, 2002). The *Zymonas mobilis* DnaK which was able to reverse the thermosensitivity of *E. coli* deficient cells is 80 % identical to *E. coli* DnaK (Michel, 1993). Whilst a close sequence similarity to *E. coli* DnaK seems an obvious requirement for an exogenous DnaK to functionally replace DnaK in complementation assays, this is not always the case. For example, although *Vibrio harveyi* DnaK and *E. coli*

DnaK share 81.2 % identity, the former's DnaK was not able to protect *E. coli* DnaK deficient cells from heat stress (Klein *et al.*, 1998). For the same reason, a number of Hsp70s from other prokaryotic organisms were not able to functionally substitute for DnaK *in vivo*. Amongst the Hsp70s that failed to reverse thermosensitivity of *E. coli dnaK* mutant or minus strains are the following: *E. coli* Hsc62 (Arifuzzaman *et al.*, 2002); *Mycobacterium tuberculosis* DnaK (Mehlert and Young, 1989), *Bacillus megaterium* DnaK (Sussman and Setlow, 1987), and *Bacillus thermoglucosidasius* DnaK (Watanabe *et al.*, 2000). Based on the sequence alignment data previously obtained (section 2.3.1), PfHsp70 and *E. coli* DnaK share the minimum structural resemblance for possible functional overlap. It is against this background, that PfHsp70's ability to reverse the thermosensitivity phenotype when heterologously expressed in *E. coli* DnaK mutant strains was investigated.

It was hoped that the optimisation of the *E. coli* heterologous *in vivo* complementation assay would facilitate the analysis of PfHsp70 motifs that were identified as potentially crucial for its function. This chapter covers work that was conducted to investigate the role of residues V401 and Q402 in the linker of PfHsp70. Because PfHsp70 possesses a conserved linker region closely resembling that of DnaK (section 2.3.1), it was interesting to find out if substitutions in this motif of PfHsp70 would influence its activity. It has been established that the mechanism of action of Hsp70 involves interdomain communication between the ATPase and the peptide binding domains (Slepenkov and Witt, 2003) and the highly conserved linker region is important for this interaction (Han and Christen, 2001). The disruption of interdomain coupling in DnaK by mutating linker residues also interferes with the DnaJ regulated activity of DnaK (Laufen *et al.*, 1999). Thus, Hsp70 interdomain coupling is essential, not only for the modulation of Hsp70 by Hsp40, but for regulation of its key functional aspects such as ATPase activity and substrate affinity as well (Laufen *et al.*, 1999; Moro *et al.*, 2003; Slepenkov and Witt, 2003). In addition, the lid region (α-helical A and half of α-helical B subdomains) is also involved in the interdomain coupling of DnaK (Moro *et al.*, 2003). The fact that the regulation of the allosteric function of Hsp70 is regulated through several motifs on its structure, highlights the importance of this process in the functional cycle of Hsp70.

The objectives of this study were to:

1. Establish PfHsp70s ability to reverse the thermo-sensitivity of *E. coli* DnaK mutant cells.
2. Investigate the functional compatibility between the ATPase and peptide binding domains of PfHsp70 and *E. coli* DnaK by swapping the two domains.
3. Study the role of some residues (V401 and Q402) in the linker region.
4. Carry out expression studies in *E. coli* DnaK mutant strains for PfHsp70, DnaK and their mutant derivatives used in this study.

3.2 Experimental procedures

3.2.1 Materials, special reagents and kits

The following are the plasmids that were used in this study: pQE30 plasmid vector (Qiagen, Germany); pQE60 (originally from Qiagen, Germany) and pBB46 (pQE60/DnaK) plasmids were a kind donation from Dr. W. Burkholder (Stanford University, U.S.A) and. For the expression of PfHsp70, the pQE30/PfHsp70 plasmid (Matambo *et al.*, 2004) was used. This plasmid is a derivative of the original pRSETB/PfHsp70 (Kumar and Zheng, 1992; Matambo *et al.*, 2004) which was donated to our laboratory by Dr. N. Kumar, John Hopkins University, U.S.A). The *PFHSP70* gene that was mounted on this plasmid was derived from *P. falciparum*, strain 3D7, isolate NF54 (Kumar and Zheng, 1992). The rest of the materials and special reagents used in this study are provided in (Table D.1; Appendix D).

3.2.2 Confirmation of the pQE30/PfHsp70 and pQE60/DnaK (pBB46) plasmid by restriction analysis

In order to confirm the integrity of the pQE30/PfHsp70 plasmid construct, the plasmid was purified using the Qiagen (Germany) DNA minipreparation kit following the instructions of the supplier (Appendix A.3). The DNA was digested (Appendix A.5) using the diagnostic restriction enzymes, *Hind* III and *Bam* HI. The products were then analysed by agarose gel electrophoresis (Appendix A.4) in order to estimate the size of the fragments. The status of the pQE60/DnaK plasmid was confirmed by restricting purified plasmid DNA using the diagnostic enzyme, *Pvu* I. Since there was no *Afl* II site in pQE60/DnaK plasmid, this enzyme was used to confirm the exclusion of this site in this plasmid, prior to the introduction of another *Afl* II site in the linker encoding segment for domain swapping work.

3.2.3 Construction of expression plasmids encoding chimeric proteins

In order to facilitate domain swapping between PfHsp70 and DnaK, an *Afl* II restriction site was introduced into the coding regions of the linker region of both PfHsp70 and DnaK following the strategy that was used by other workers (Mogk *et al.*, 1999; Suppini *et al.*, 2004). The *Afl* II restriction site facilitated the construction of two plasmids: one encoding the ATPase domain of PfHsp70 fused to the peptide binding domain of DnaK (PfK); and the second encoding the ATPase domain of DnaK fused to the peptide binding domain of PfHsp70 (KPf).

3.2.3.1 Introduction of a common *Afl* II restriction site in plasmids pQE30/PfHsp70 and pQE60/DnaK for domain swapping

An existing *Afl* II restriction site was eliminated from the coding region of PfHsp70 to facilitate the construction of the plasmids encoding chimeric proteins. Site-directed mutagenesis was carried out by designing a primer that eliminated the *Afl* II restriction site (Table B.1; Appendix B), introducing a silent mutation on the pQE30/PfHsp70 plasmid to give rise to plasmid pQE30/PfHsp70-EL (Figure 3.1). *Afl* II restriction sites were introduced into the coding regions of the linker region of both PfHsp70 and DnaK to facilitate the construction of the plasmids encoding chimeric proteins (Figure 3.1). Two amino acid substitutions were generated (V401L and Q402K) as a result of the re-introduction of another *Afl* II restriction site in pQE30/PfHsp70-EL to produce the plasmid pQE30/PfHsp70-V401L/Q402K, encoding the protein PfHsp70-V401L/Q402K. The *Afl* II restriction site that was introduced in plasmid pQE30/PfHsp70-EL was located in the segment encoding the linker (Figure 3.1). The pQE60/DnaK plasmid served as template for the introduction of the *Afl* II site by site-directed mutagenesis. The introduction of the *Afl* II restriction site accounted for the V386L conservative amino acid change in DnaK (to give the modified protein DnaK-V386L). The QuikChange site-directed mutagenesis kit from Stratagene (U.S.A) was employed to introduce the restriction sites for the domain swapping work, based on the standard protocol provided by the supplier (Appendix A.6). Primers synthesised by IDT, U.S.A.(Table B.1; Appendix B), were used for site-directed mutagenesis. Figure 3.1 summarises the strategy that was used for the domain swapping work.

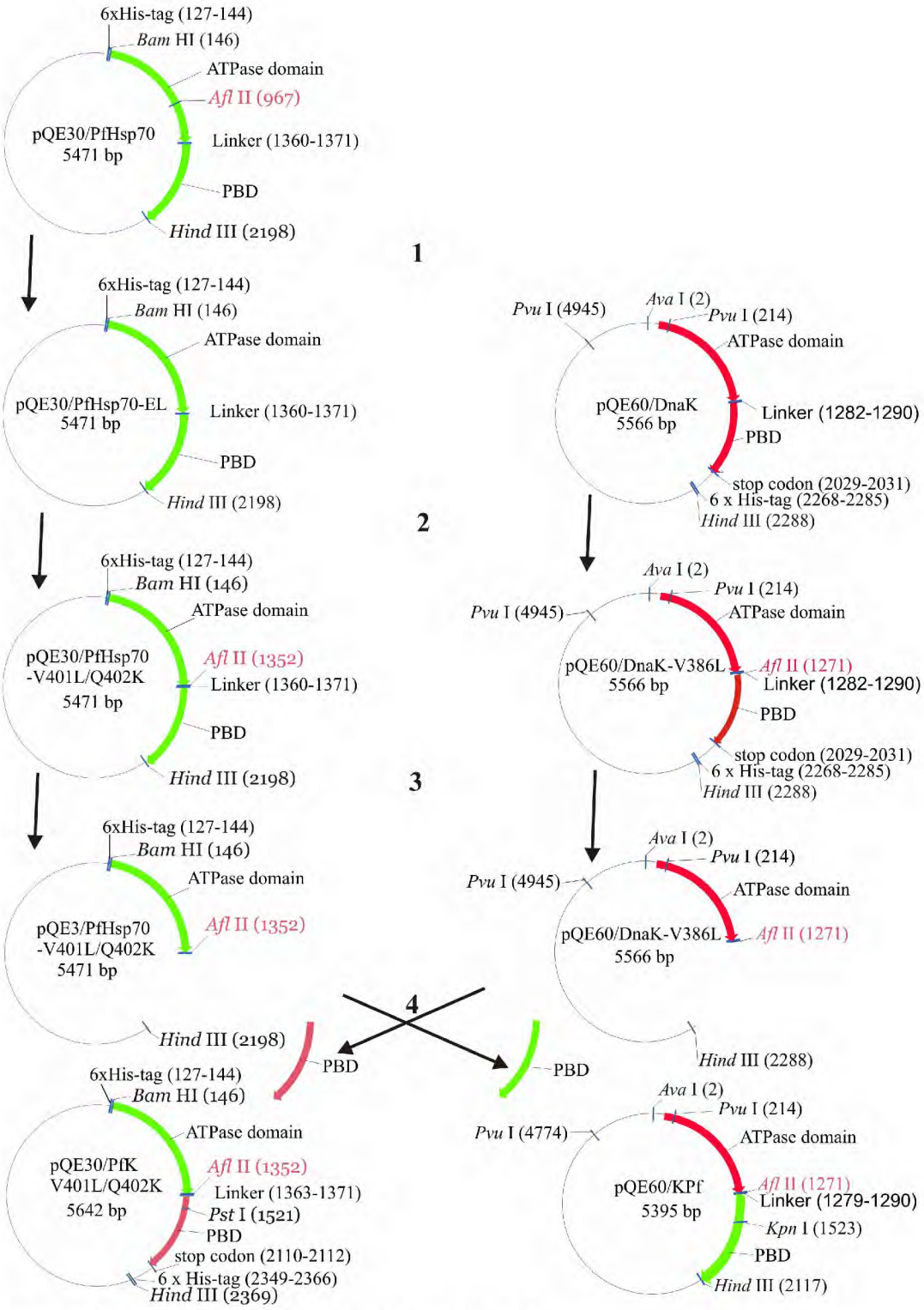

Figure 3.1 Strategy for the construction of chimeric proteins
(1) Elimination of the *Afl* II restriction site in pQE30/PfHsp70 to get pQE30/PfHsp70-EL, (2) Introduction of *Afl* II restriction sites in pQE30/PfHsp70-EL and pQE60/DnaK plasmids to yield pQE30/PfHsp70-V401L/Q402K and PQE60/DnaK-V386L plasmids, respectively, (3) peptide binding domain (PBD) coding regions were gel purified, and (4) PBD coding fragments were swapped around and ligated to form plasmids: pQE30PfK-V401L/Q402K and pQE60/KPf, encoding for chimeric proteins PfK-V401L/Q402K and KPf, respectively.

3.2.3.2 Construction of the plasmids encoding chimeric proteins KPf and PfHsp70-V401L/Q402K

By taking advantage of the common *Afl* II restriction site that was introduced in the plasmids encoding both PfHsp70 and *E. coli* DnaK, the two plasmids were restricted by *Afl* II and *Hind* III, thus releasing the segment encoding the peptide binding domains of both proteins (Figures 3.1). The DNA segments encoding the peptide binding domains of both proteins were gel purified using an Amersharm (U.S.A) kit whose protocol is provided (Appendix A.7). The purified DNA fragments were then swapped around and fused back to opposite plasmid vectors and ligated, yielding plasmids pQE30/PfK-V401L/Q402K (encoding chimeric protein, PfK-V401L/Q402K) and pQE60/KPf (encoding chimeric protein, KPf), respectively.

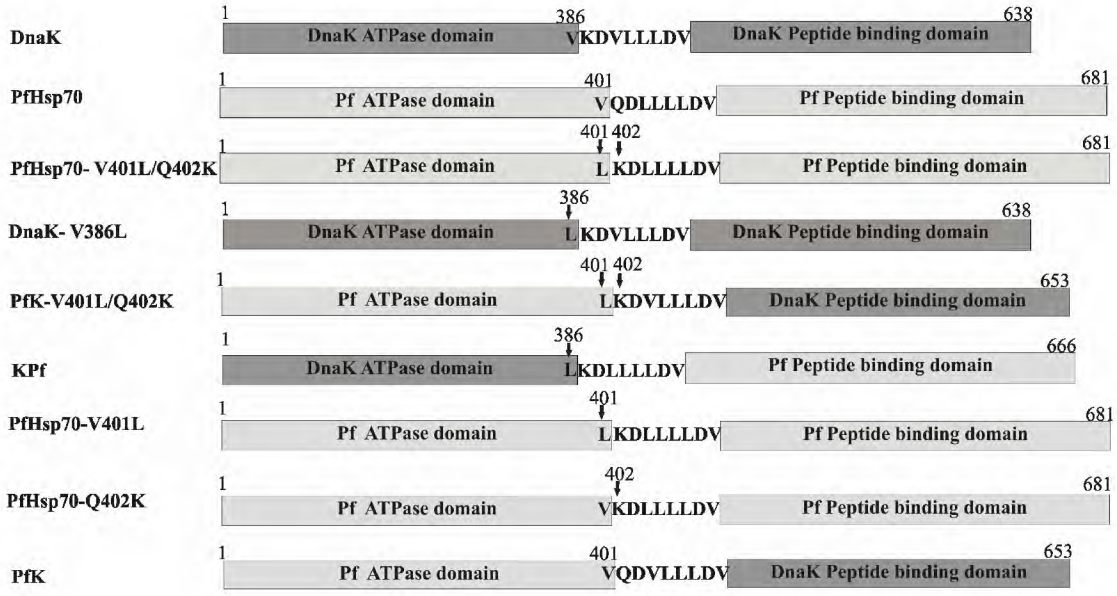

Figure 3.2 Chimeric proteins derived from domains of PfHsp70 and *E. coli* DnaK
An *Afl* II site was introduced in the sequence encoding the linker region between the ATPase and peptide binding domains of PfHsp70 and DnaK using site-directed mutagenesis. This restriction site was used to link the ATPase domain coding region of PfHsp70 with the peptide binding domain coding region of DnaK and vice versa, resulting in constructs encoding the chimeric proteins PfK-V401L/Q402K and KPf, respectively. The introduction of the *Afl* II site resulted in a conservative amino acid change (V386L) in DnaK to give DnaK-V386L, and two amino acid changes in PfHsp70 (V401L and Q402K) to give PfHsp70-V401L/Q402K. Reversion of each of the codon changes in PfHsp70 gave rise to the constructs encoding PfHsp70-V401L, PfHsp70-Q402K and PfK

3.2.3.3 Reverse changes introduced on PfHsp70-V401L/Q402K and PfK-V401L/Q402K

The introduction of the *Afl* II restriction site generated a derivative of PfHsp70 (PfHsp70-V401L/Q402K) and consequently chimera PfK-V401L/Q402K. In order to examine the effect of the amino acids substitutions on the complementation ability of PfHsp70, reverse mutations were made on pQE30/PfHsp70-V401L/Q402K, resulting in the construction of plasmids pQE30/PfHsp70-V401L and pQE30/PfHsp70-Q402K, encoding the proteins PfHsp70-V401L and PfHsp70-Q402K (Figure 3.2). A double reversion of the amino acid substitutions in the chimera PfK-V401L/Q402K gave rise to the plasmid, pQE30/PfK, encoding PfK (Figure 3.2).

3.2.4 Complementation assays

Three different thermosensitive *E. coli* strains were used for complementation assays in this study (Table 3.1). A DnaK deletion strain, *E. coli* Δ*dnaK52*, BB1553 (MC4100 Δ*dnaK52*::*Cm*^{R} *sidB1*), has a *dnaK* gene that is disrupted by a *cat* cassette, resulting in a thermosensitive phenotype (Paek and Walker 1987). This strain grows normally at 30°C and is sensitive to both heat and cold (Bukau and Walker 1989, 1990). *E. coli dnaK103*, BB2393 (C600 *dnaK103 thr*::*Tn10*::*Tet*^{R}) strain has an amber mutation in the *dnaK* gene and produces a truncated, non-functional DnaK (Spence *et al.*, 1990). Temperatures of growth for this strain are the same as those for the *E. coli* Δ*dnaK52* strain. However, *E. coli* Δ*dnaK52* strain is characterised by reduced DnaJ levels due to the polar effect of the *Cm*^{R} cassette situated downstream of the *dnaJ* gene (Sell *et al.*, 1990). Therefore use of the *E. coli dnaK103* strain for complementation studies is recommended because this strain has a non-functional DnaK and possesses sufficient levels of DnaJ to meet physiological requirements (Spence *et al.*, 1990; Suppini *et al.*, 2004). The *E. coli dnaK756*, BB2362 (*dnaK756 recA*::*Tc*^{R} *pDMI,1*) strain is resistant to bacteriophage lambda (Georgopoulos, 1977), and is unable to grow above 40°C (Georgopoulos *et al.*, 1979; Tilly *et al.*, 1983). This strain expresses a DnaK with three amino acid substitutions, one of which reduces its affinity for GrpE, whilst the two other substitutions elevate the basal ATPase activity of DnaK (Buchberger *et al.*, 1999).

The cells were transformed (Appendix A.2) using plasmids encoding the different Hsp70s before being subjected to stress temperatures in order to monitor the capabilities of the respective proteins to reverse the thermosensitivity of the cells. The following plasmids were used to transform cells for the different *E. coli* strains: pQE30, pQE30/PfHsp70,

pQE30/PfHsp70-V401L/Q402K, pQE30/PfHsp70-V401L, pQE30/PfHsp70-Q402K, pQE30/PfK, pQE60, pQE60/KPf, pBB46 (pQE60/DnaK) and pQE60/DnaK-V386L. Freshly transformed *E. coli* Δ*dnaK52* and *dnaK103* cells were grown overnight at 30°C in double-strength yeast-tryptone broth (2xYT) cultures containing 34 μg/ml chloramphenicol and 10 μg/ml tetracycline for the respective strain as well as 100 μg/ml ampicillin for plasmid selection. The overnight inoculum was transferred into fresh broth and incubated under the same growth conditions. At mid-log phase of growth, some cells were induced with 1 mM IPTG whilst others were not. The cells were left to grow to 2.0 A_{600} (2.0 absorbance units at 600 nm). The cultures were standardised to a cell density of 0.2 A_{600}, and diluted before being spotted onto 2xYT agar plates containing the necessary antibiotics and 50 μM IPTG, and incubated overnight at 30°C and 40°C. The *E. coli dnaK756* cells were also treated the same way except that the strain was selected for using 50 μg/ml kanamycin and 10 μg/ml tetracycline. The liquid culture was grown at 37°C prior to standardisation and spotting on 2xYT agar plates containing the necessary antibiotics and 20 μM IPTG. The plates were then incubated overnight at 37°C and 43.5°C.

Table 3.1 *E. coli* strains used for complementation assays

Name of *E. coli* strain	Characteristics	P G T	N P G T	Antibiotics	References
BB1553 (MC4100 Δ*dnaK52*::Cm^R *sidB1*)	-*dnaK* gene substituted with *cat* cassette -low DnaJ levels	30°C	40°C	chloramphenicol tetracycline	-Paek and Walker (1987) -Bukau and Walker, (1989b) -Bukau and Walker, 1990 -Sell *et al.* (1990)
BB2393 (C600 *dnaK103* *thr*::*Tn10*::Tet^R)	-expresses a truncated DnaK -high levels of DnaJ	30°C	40°C	chloramphenicol tetracycline	-Spence *et al.* (1990) - Mayer *et al.* (2000b)
BB2362 (*dnaK756* *recA*::Tc^R *pDMI,1*)	-expresses the DnaK756 protein which has reduced GrpE affinity and elevated basal ATPase activity -normal DnaJ levels	30°C - 37°C	>40°C	Kanamycin Tetracycline	-Georgopoulos (1977) -Georgopoulos *et al.* (1979) -Tilly *et al.* (1983) -Buchberger *et al.* (1999)

Permissive Growth Temperatures (PGT) and Non-Permissive Growth Temperatures (NPGT)

3.2. 5 Protein expression studies

Flasks containing 2xYT broth (10 ml) with the necessary antibiotics added were prepared, before introducing cells transformed with the plasmids encoding different proteins. The *E. coli dnaK103* and *E. coli dnaK52* transformants were incubated at 30°C whilst the *E. coli dnaK756* transformants were incubated at 37°C, overnight. The cells that were grown overnight (10 ml culture) were then used to inoculate 90 ml of fresh 2xYT broth containing the respective antibiotics. Cultures were left to grow to mid-log phase before being induced with 1mM IPTG. Samples were collected for the first 5 hours for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (Appendix A.8). The production of the His-tagged proteins and DnaK was confirmed by Western analysis (Appendix A.9; A.10) using anti-His antibodies (Amersham-Pharmacia Biotech, Piscataway, N.J, U.S.A) and monoclonal anti-*E. coli* DnaK antibodies (Stressgen Biotechnology, U.S.A), respectively. Chemiluminescent immunodetection was carried out on Western blots as described (Appendix A.10) and visualised using the Bio-Rad's VersaDoc™ Model 4000 imaging system.

3.3 Results

3.3.1 Confirmation of pQE30/PfHsp70 and the elimination of its *Afl* II restriction site

Diagnostic endonuclease restriction analysis was carried out on the pQE30/PfHsp70 plasmid (Figure 3.3A; Matambo *et al.*, 2004). The plasmid had an original *Afl* II site in the ATPase domain that was eliminated by site-directed mutagenesis (Figure 3.3B). The resultant silent mutation was also confirmed by DNA sequencing.

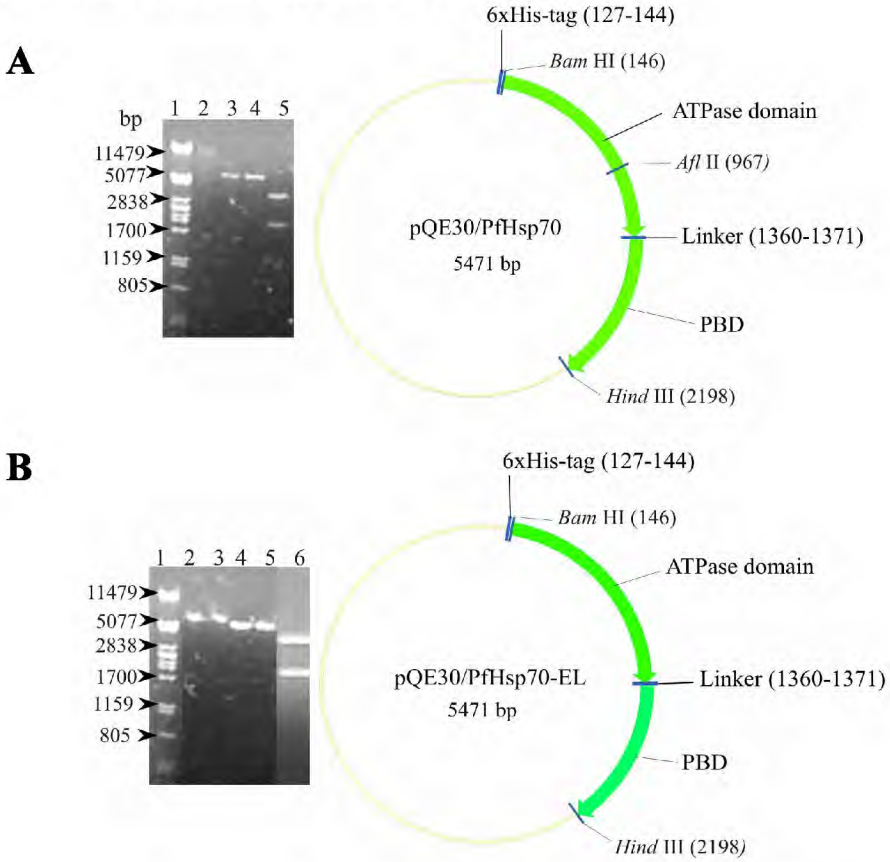

Figure 3.3 Diagnostic restriction analysis of pQE30/PfHsp70 and pQE30/PfHsp70-EL
Ethidium bromide stained agarose gels and restriction maps of pQE30/PfHsp70 (**A**) and (**B**) pQE30/PfHsp70-EL. The DNA was loaded onto the gel in the following order: (A) lane 1, lambda DNA molecular markers digested with *Pst* I; lane 2, undigested pQE30/PfHsp70 construct; lane 3, pQE30/PfHsp70 construct digested with *Afl* II; lane 4, pQE30/PfHsp70 construct digested with *Hind* III; lane 5, pQE30/PfHsp70 construct digested with *Hind* III and *Bam* HI. (**B**) Lane 1, lambda DNA molecular weight standards digested with *Pst* I; lane 2, pQE30/PfHsp70-EL undigested plasmid; lane 3, pQE30/PfHsp70-EL digested with *Afl* II; lane 4, pQE30/PfHsp70-EL digested with *Hind* III; lane 5, pQE30/PfHsp70-EL digested with *Afl* II and *Hind* III and lane 6, pQE30/PfHsp70-EL digested with *Bam* HI and *Hind* III. The regions on the plasmids encoding for the ATPase domain, the linker region and binding domain (PBD) of PfHsp70 are highlighted. Also shown is the N-terminal His-tag encoding segment.

3.3.2 Introduction of an *Afl* II restriction site into pQE30/PfHsp70 for facilitating domain swapping

The re-introduction of the *Afl* II restriction site into the pQE30/PfHsp70-EL plasmid was confirmed by agarose gel analysis (Figure 3.4). The new *Afl* II restriction site was introduced in the segment encoding the linker region of PfHsp70. The codon changes caused amino acid changes on PfHsp70, giving rise to the modified protein, PfHsp70-V401L/Q402K.

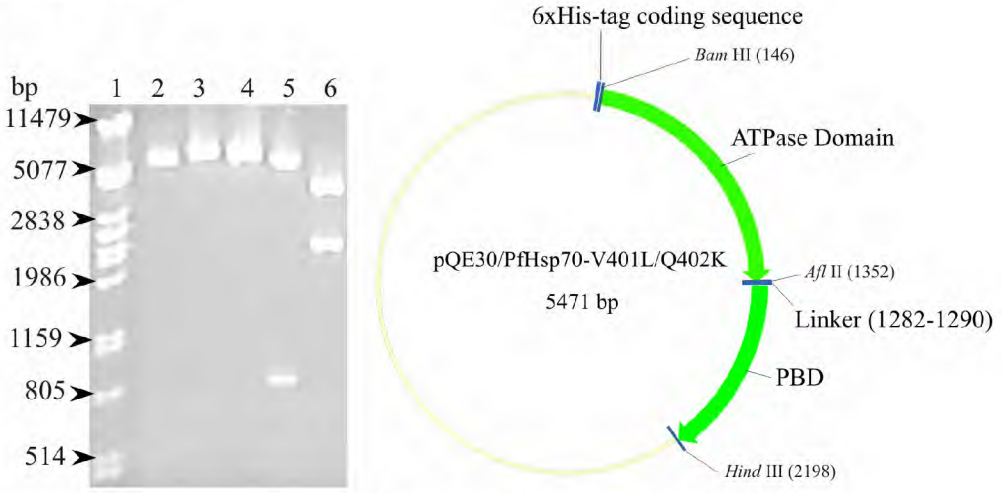

Figure 3.4 Diagnostic restriction analysis of pQE30/PfHsp70-V401L/Q402K
Ethidium bromide stain of 0.8% agarose gel and restriction map of the pQE30/PfHsp70-V401L/Q402K plasmid. Lane 1, *Pst* I digested lambda DNA molecular weight standards; lane 2, pQE30/PfHsp70-V401L/Q402K undigested; lane 3, pQE30/PfHsp70-V401L/Q402K digested with *Hind* III; lane 4, pQE30/PfHsp70-V401L/Q402K digested with *Afl* II; lane 5, pQE30/PfHsp70-V401L/Q402K digested with *Afl* II and *Hind* III; lane 6, pQE30/PfHsp70–V401L/Q402K digested with *Hind* III and *Bam* HI. The regions encoding for the ATPase domain, the linker region and binding domain (PBD) of PfHsp70 are highlighted. Also shown is the N-terminal His-tag encoding segment.

3.3.3 Analysis of the pBB46 (pQE60/DnaK) construct and introduction of the *Afl* II restriction site to facilitate domain swapping

The absence of an *Afl* II site in pQE60/DnaK was confirmed by the fact that *Afl* II was not able to cut this plasmid (lane 3, Figure 3.5 A). Digestion of pQE60/DnaK using *Pvu* I was conducted for further diagnostic restriction analysis. To facilitate the swapping of the coding regions for the ATPase and peptide binding domains of PfHsp70 and DnaK, an *Afl* II restriction site was engineered into the pQE60/DnaK plasmid encoding DnaK (Figure 3.5B). The introduction of the *Afl* II restriction site accounted for the codon changes which caused the V386L conservative amino acid change in DnaK (resulting in the modified protein DnaK-V386L). The introduction of the *Afl* II site was confirmed by diagnostic restriction analysis (lanes 4-7; Figure 3.5B). The fact that a stop codon is situated upstream

of the His-tag coding region implies that the DnaK protein encoded by both pQE60/DnaK and pQE60/DnaK-V386L does not have a C-terminal His-tag (Figure 3.5).

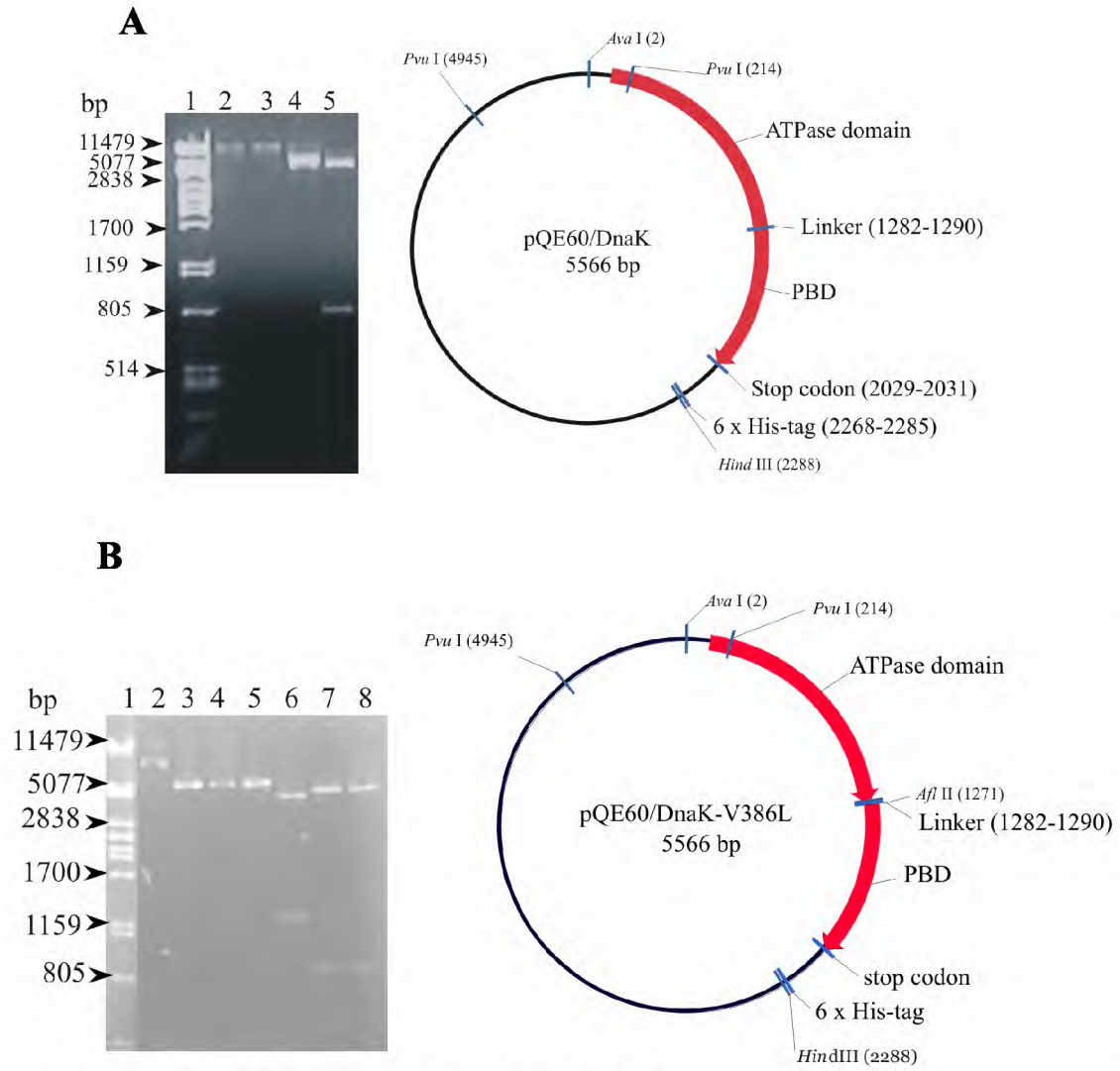

Figure 3.5 Diagnostic restriction of pQE60/DnaK and pQE60/DnaK-V386L
Ethidium bromide stain of 0.8% agarose gels and restriction maps of the pQE60/DnaK (A) and pQE60/DnaK-V386L (B) plasmids, respectively. (**A**) Lane 1, *Pst* I digested lambda DNA molecular markers; lane 2, pQE60/DnaK d undigested; lane 3, pQE60/DnaK digested with *Afl* II; lane 4, pQE60/DnaK digested with *Hind* III, and lane 5, pQE60/DnaK digested with *Pvu* I. (**B**) Lane 1, lambda DNA molecular weight standards digested with *Pst* I; lane 2, pQE60/DnaK-V386L undigested; lane 3, pQE60/DnaK-V386L digested with *Hind* III; lane 4, pQE60/DnaK-V386L digested with *Afl* II; lane 5, pQE60/DnaK-V386L digested with *Ava* I; lane 6, pQE60/DnaK-V386L digested with *Ava* I and *Afl* II; lane 7, pQE60/DnaK-V386L digested with *Hind* III and *Afl* II, and lane 8, pQE60/DnaK-V386L digested with *Pvu* I. The regions encoding the ATPase domain, the linker region and the peptide binding domain (PBD) are highlighted. A stop codon is located just after the DnaK coding region and before the C-terminal His-tag coding region (6 x His-tag).

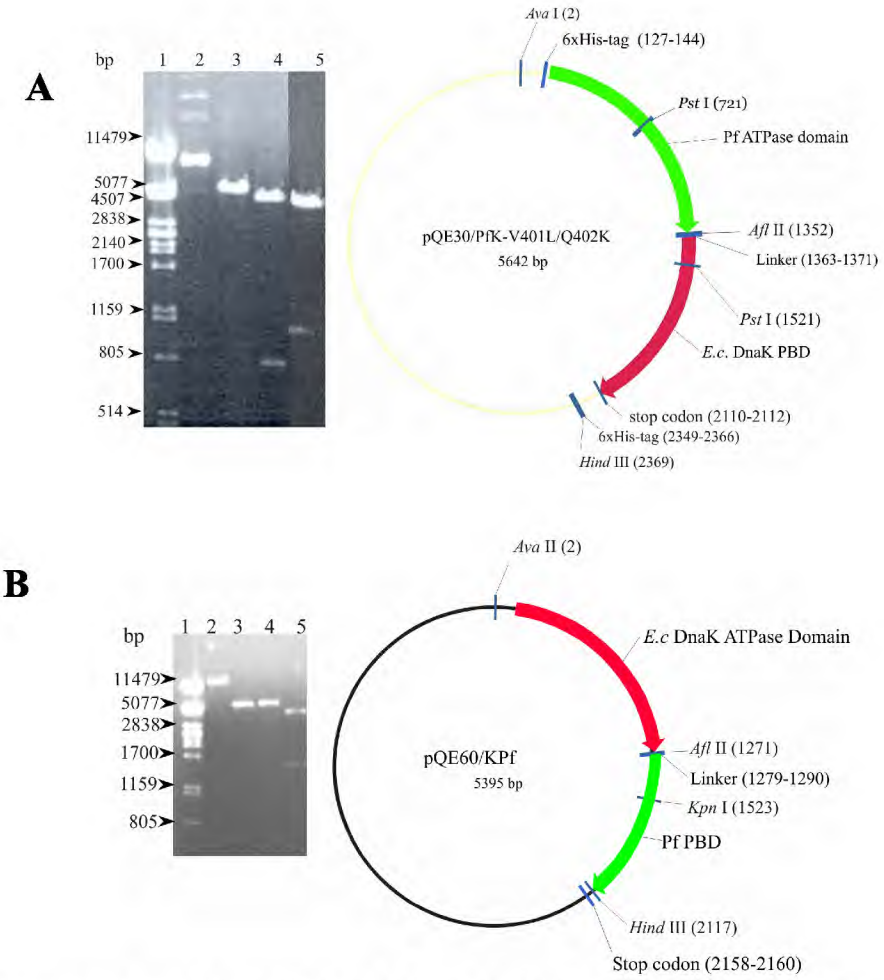

Figure 3.6 Diagnostic restriction analysis of pQE30/PfK-V401L/Q402K and pQE60/KPf
Ethidium bromide stain of 0.8% agarose gels and restriction maps for the pQE30/PfK-V401L/Q402K (**A**) and pQE60/KPf (**B**) plasmids. (**A**) Lane 1, *Pst* I digested lambda DNA molecular weight standards; lane 2, pQE30/PfK-V401L/Q402K undigested; lane 3, pQE30/PfK-V401L/Q402K digested with *Ava* I; lane 4, pQE30/PfK-V401L/Q402K digested with *Pst* I; lane 5, pQE30/PfK–V401L/Q402K digested with *Hind* III and *Afl* II. The PfHsp70 ATPase domain (Pf ATPase domain) and *E. coli* DnaK peptide binding domain (E.c PBD) encoding regions are shown linked through the linker encoding segment (linker). (**B**) Lane 1, *Pst* I digested lambda DNA molecular weight standards; lane 2, pQE60/KPf udigested; lane 3, pQE60/KPf digested with *Ava* I; lane 4, pQE60/KPf digested with *Kpn* I; and lane 5, pQE60/KPf digested with *Ava* I and *Kpn* I. The KPf protein is encoded by the highlighted *E. coli* DnaK ATPase (E.c DnaK ATPase domain) and the PfHsp70 peptide binding domain (PfPBD) coding regions, fused through the linker encoding segment (linker).

3.3.4 Construction of the plasmid constructs encoding chimeric proteins PfK-V401L/Q402K and KPf

The generation of plasmids pQE30/PfHsp70-V401L/Q402K and pQE60/DnaK-V386L with a common *Afl* II site in the linker encoding segment (Figures 3.4 and 3.5B), enabled the swapping of coding regions for the domains of these two proteins (Figure 3.6). Thus, the plasmid expression constructs were generated that encoded the *E. coli* DnaK ATPase domain fused to the PfHsp70 peptide binding domain (called KPf), and the PfHsp70 ATPase domain

fused to the *E. coli* DnaK peptide binding domain (called PfK-V401L/Q402K). The resultant plasmids pQE30/PfK-V401L/Q402K (encoding chimeric protein, PfK-V401L/Q402K) and pQE30/KPf (encoding chimeric protein, KPf) were confirmed by both restriction analysis (Figure 3.6) and by DNA sequencing (Appendix A.11). *Pst* I was used as the key diagnostic restriction endonuclease for the pQE30/PfK-V401L/Q402K (Figure 3.6A). *Kpn* I and *Ava* I were used for restriction analysis to confirm the status of the pQE30/KPf plasmid (Figure 3.6B). The chimeric proteins encoded by the respective plasmids carried amino acid changes introduced in the linker regions that were attached to the ATPase domains of their parental proteins (KPf had a V386L substitution, whilst PfK-V401L/Q402K carried the V401L/Q402K double substitution).

3.3.5 Construction of reversion plasmids, pQE30/PfHsp70-V401L, pQE30/PfHsp70-Q402K and PfK

Reversion mutations that were conducted using the pQE30/PfHsp70-V401L/Q402K plasmid as template DNA in order to construct plasmids encoding mutants PfHsp70-V401L and PfHsp70-Q402K were confirmed by restriction analysis (Figure 3.7A) as well as DNA sequencing (Appendix A.11). The *Afl* II restriction site that was used to facilitate domain swapping was eliminated as a result of these changes (Figure 3.7A). Similarly, plasmid pQE30/PfK-V401L/Q402K (Figure 3.6A) was changed to produce plasmid pQE30/PfK (which encoded the chimera PfK that had the double substitution in the linker reversed; Figure 3.7B). The elimination of the *Afl* II site in pQE30/PfHsp70-Q402K, confirmed the reversion of pQE30/PfHsp70-V401L/Q402K to pQE30/PfHsp70-Q402K (lanes 3-5; Figure 3.7A). Similarly, the elimination of the *Afl* II site from pQE30/PfK-V401L/Q402K upon its conversion to pQE30/PfK-V401L was confirmed through diagnostic restriction analysis (lanes 9-10; Figure 3.7A). The conversion of pQE30/PfK-V401L/Q402K to pQE30/PfK also resulted in elimination of the *Afl* II restriction site (lanes 3-4; Figure 3.7B). All the changes were confirmed by DNA sequencing (Appendix A.11).

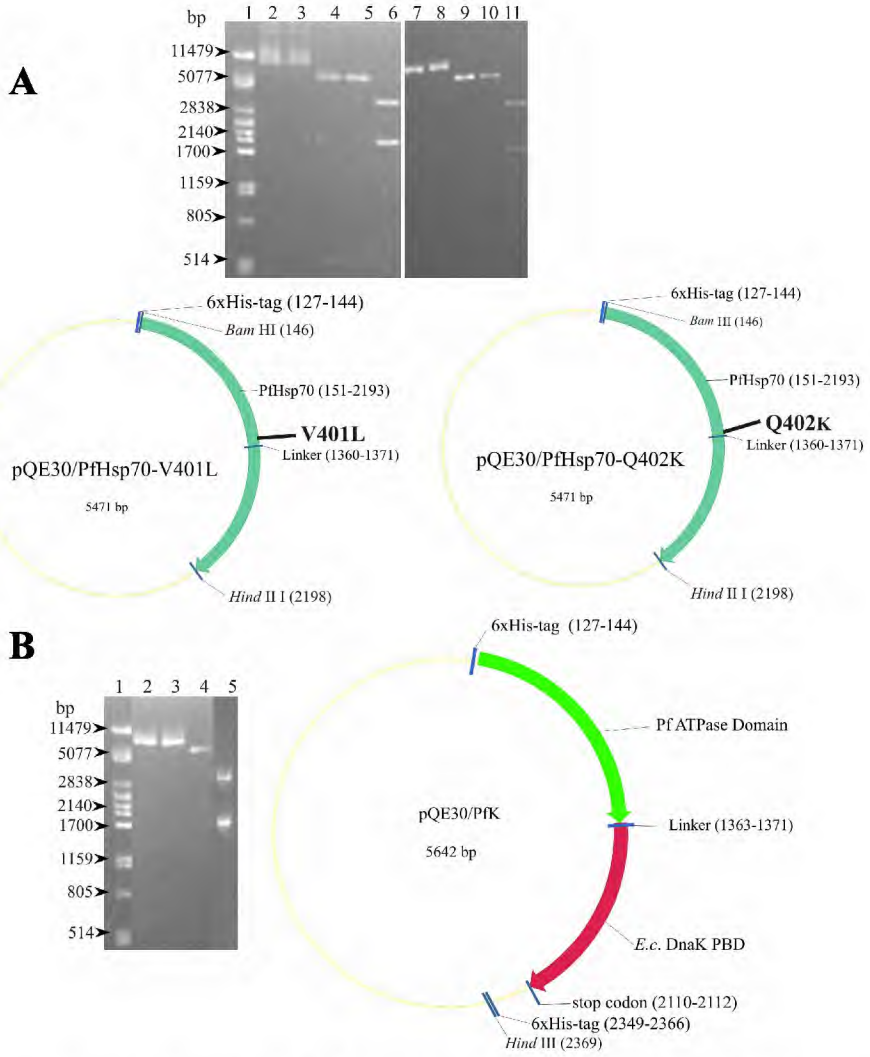

Figure 3.7 Diagnostic restriction analysis of pQE30/PfHsp70-V401L, pQE30/PfHsp70-Q402K and PfK
Ethidium bromide stain of 0.8% agarose gels and restriction maps for the pQE30/PfHsp70-V401L, pQE30/PfHsp70-Q402K (**A**) and pQE30/PfK (**B**) plasmids, respectively. (**A**) Lane 1, *Pst* I digested lambda DNA molecular weight standards; lane 2, pQE30/PfHsp70-V401L undigested; lane 3, pQE30/PfHsp70-V401L digested with *Afl* II; lane 4, pQE30/PfHsp70-V401L digested with *Hind* III; lane 5, pQE30/PfHsp70-V401L digested with *Hind* III and *Afl* II; lane 6, pQE30/PfHsp70-V401L digested with *Hind* III and *Bam* HI; Lane 7, pQE30/PfHsp70-Q402K undigested; lane 8, pQE30/PfHsp70-Q402K digested with *Afl* II; lane 9, pQE30/PfHsp70-Q402K digested with *Hind* III; lane 10, pQE30/PfHsp70-Q402K digested with *Hind* III and *Afl* II; lane 11, pQE30/PfHsp70-Q402K digested with *Hind* III and *Bam* HI. The position of the V401L and Q402K encoding sites for the two plasmids pQE30/PfHsp70-V401L and pQE30/PfHsp70-Q402K plasmids are highlighted close to the linker encoding site. (**B**) Lane 1, *Pst* I digested lambda DNA molecular weight standards; lane 2, pQE30/PfK undigested; lane 3, pQE30/PfK digested with *Afl* II; lane 4, pQE30/PfK digested with *Hind* III and *Afl* II; lane 5, *Hind* III and *Bam* HI. The PfHsp70 ATPase domain (Pf ATPase domain) and *E. coli* DnaK peptide binding domain (E.c PBD) encoding regions are shown linked through the linker encoding segment (linker).

3.3.6 Complementation assays

Results obtained for the complementation assays based on studies conducted using the *E. coli* Δ*dnaK52* and *dnaK103* strains were the same with or without pre-inducing the cells using IPTG during the broth culture stage prior to spotting onto agar plates. However, when *E. coli dnaK756* transformants grown to the mid-log phase were induced using 1 mM IPTG (prior to the plating stage), they were able to express higher levels of PfHsp70 and KPf. The *E. coli dnaK756* cells expressing these proteins were able to grow at 43.5°C (Figure 3.10A). Data reported here for all the *E. coli* strains used, was based on the broth cultures that were induced before the plating stage. However, IPTG induction whilst improving the heterologous expression of the proteins, also led to production of toxic levels of DnaK (Figures 3.8; 3.9 and 3.10).

3.3.6.1 Complementation assay in *E. coli* Δ*dnaK52* strain

All the *E. coli* Δ*dnaK52* cells transformed with the different plasmids were able to grow at 30°C (Figure 3.8A). Since *E. coli* Δ*dnaK52* cells can grow at this temperature (Bukau and Walker, 1989a,b), the ability of these cells to grow after being transformed with the different plasmids shows that the plasmids and their products (the heterologously expressed proteins) did not induce cell toxicity. At the non-permissive temperature of 40°C, *E. coli* Δ*dnaK52* cells transformed with pQE30 and pQE60 plasmid vectors (negative controls) were not able to grow. These two plasmids were not encoding for any chaperones. Cells that were producing *E. coli* DnaK (positive control) and its variant (DnaK-V386L) from their respective plasmids were able to grow at 40°C. The production of these two proteins was confirmed by Western analysis. Although *E. coli* DnaK-V386L was able to reverse thermosensitivity of *E. coli* Δ*dnaK52* cells to the same extent as the wild type protein, the cells were able to produce much higher levels of DnaK (wild type) than DnaK-V386L. Therefore, the V386L change does not interfere with the *in vivo* DnaK function.

E. coli Δ*dnaK52* cells transformed with pQE30/PfHsp70, pQE30/PfHsp70-V401L/Q402K, and pQE30/PfK-V401L/Q402K were unable to grow at 40°C and there was no evidence for the production of the proteins encoded by these plasmids by Western analysis (Figure 3.8A). Similarly, cells transformed with pQE60/KPf failed to grow at 40°C. There was no evidence for KPf production by SDS-PAGE analysis (Figure 3.8B). Western analysis was not used to confirm the production of KPf since this protein was not His-tagged. The lethal phenotype observed for cells harbouring plasmids encoding PfHsp70, PfHsp70-V401L/Q402K, PfK-

V401L/Q402K and KPf could have been due to the lack of production of the respective proteins by the *E. coli* Δ*dnaK52* cells.

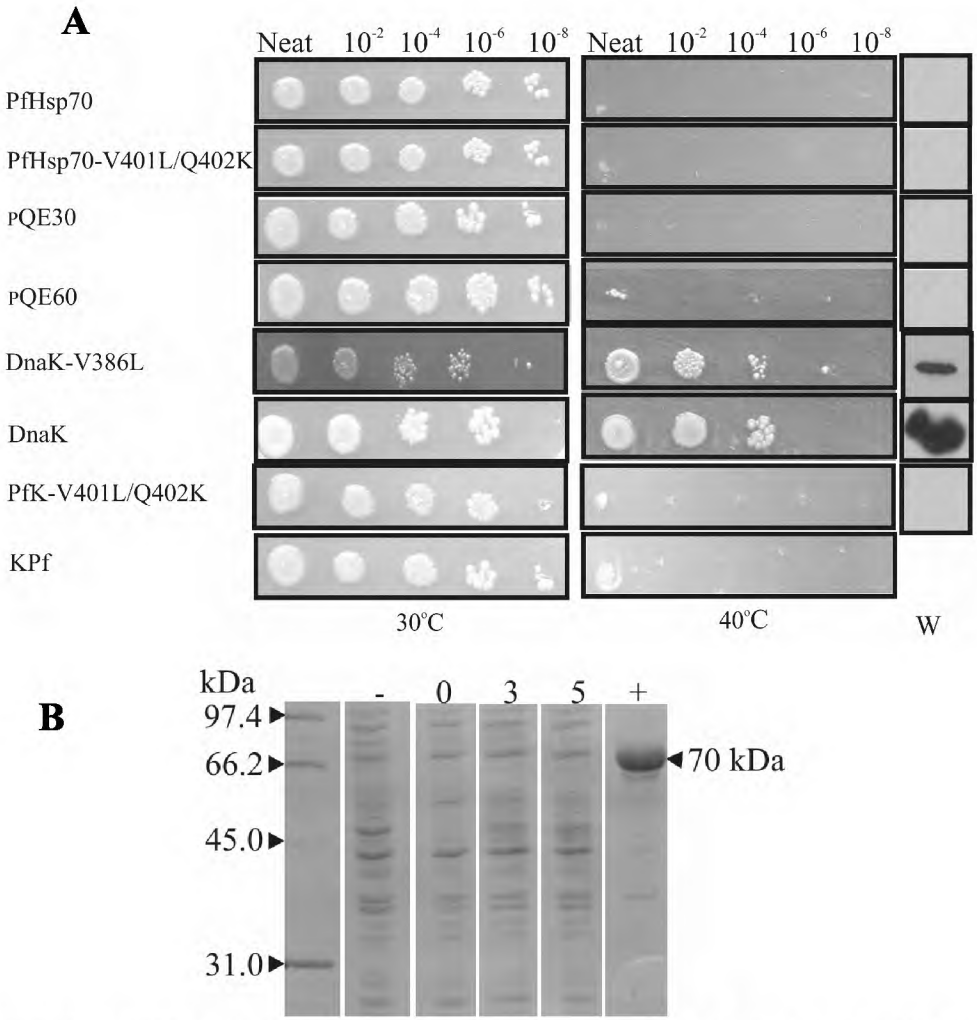

Figure 3.8 PfHsp70, PfK and KPf could not reverse the thermosensitivity of *E. coli* Δ*dnaK52* cells
(**A**) *E. coli* Δ*dnaK52* cells were transformed with plasmids pQE30/PfHsp70, pQE30/PfHsp70-V401L/Q402K, pQE30 (negative control), pQE30/PfK-V401L/Q402K, pQE60/KPf, pQE60/DnaK (positive control), pQE60/DnaK-V386L, and pQE60 (negative control). The transformants were incubated at the permissive and non-permissive temperature of 30°C of 40°C, respectively. The letter 'W' represents panels for Western analysis for the production of each protein. Western analysis of PfHsp70, PfHsp70-V401L/Q420K and PfK-V401L/Q402K was conducted using anti-His antibodies, whilst anti-*E. coli* DnaK antibodies were used to detect DnaK and DnaK-V386L. No Western analysis was conducted to detect KPf protein. (**B**) Coomasie brilliant blue stained SDS-PAGE for the detection of KPf production of chimera in *E. coli* Δ*dnaK52* cells. The lane denoted by (-) represents proteins present in total extract for cells transformed with pQE60 plasmid. Lanes 0, 3 and 5 represent proteins present in total extracts for cells transformed with pQE60/KPf, before induction with IPTG, 3 and 5 hours after induction with IPTG, respectively. The lane denoted by (+) represents purified PfHsp70 protein. The arrow on the right hand side represents mobility of a 70 kDa species. Molecular weight markers (in kDa) are shown in the extreme left hand side lane.

3.3.6.2 Complementation assay in *E. coli dnaK103* strain

At 30°C, all *E. coli dnaK103* transformants harbouring the different plasmids were able to grow (Figure 3.9A). At 40°C, cells transformed with pQE30 and pQE60 (negative controls) were unable to grow. As expected, cells transformed with pQE60/DnaK (positive control) and pQE60/DnaK-V386L were able to grow at 40°C.

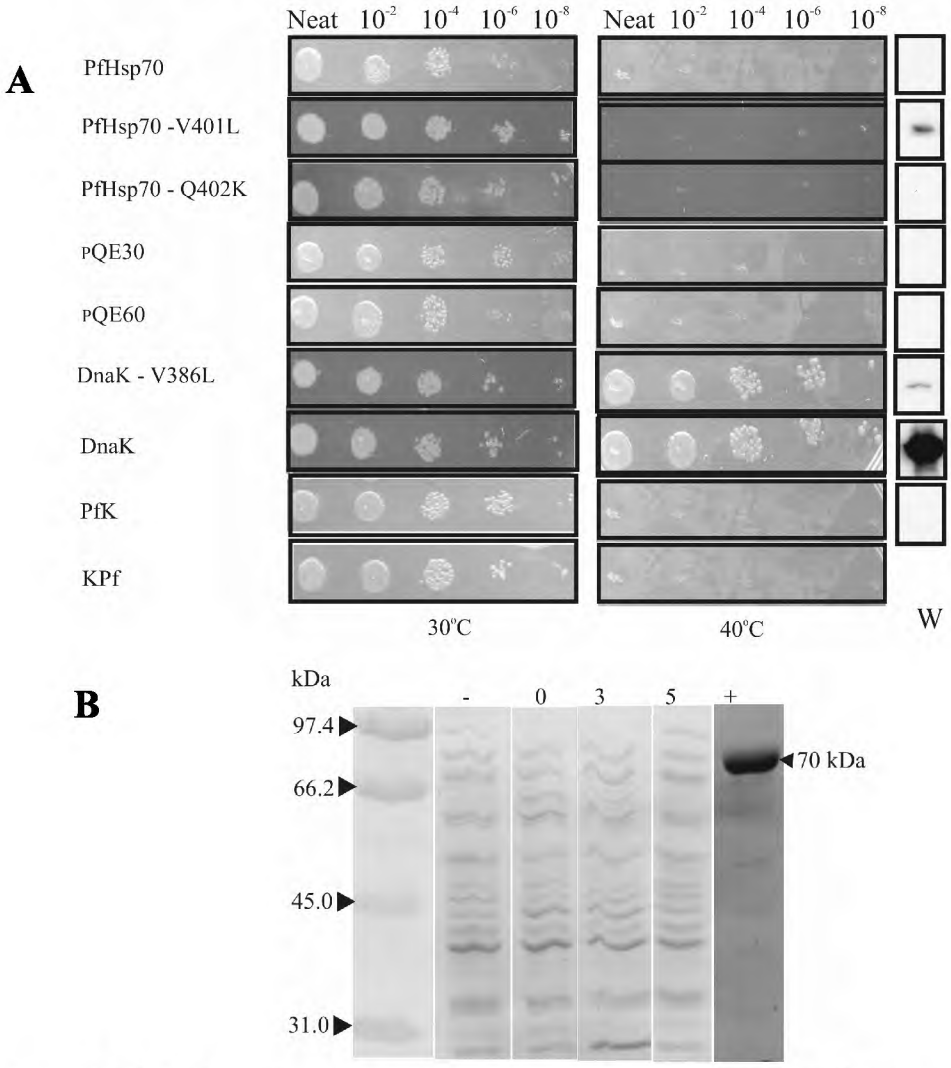

Figure 3.9 PfHsp70 was not able to reverse thermosensitivity of *E. coli dnaK103* cells
(A) *E. coli dnaK103* cells transformed with plasmids pQE30/PfHsp70, pQE30/PfHsp70-V401L, pQE30/PfHsp70-Q402K, pQE30 (negative control), pQE30/PfK, pQE60/KPf, pQE60/DnaK, pQE60/DnaK-V386L and pQE60 (negative control) were incubated at the permissive and non-permissive temperatures of 30°C and 40°C, respectively. Western analysis (data represented 'W') on PfHsp70, PfHsp70-V401L, PfHsp70-Q420K and PfK was conducted using anti-His antibodies, whilst anti-*E. coli* DnaK antibodies were used to detect DnaK and DnaK-V386L. No Western analysis was conducted to detect KPf protein. **(B)** Coomasie brilliant blue stained SDS-PAGE analysis for the detection of KPf in *E. colidnaK103* cells. The lane denoted by (-) represents proteins present in total extract for cells transformed with pQE60 plasmid. Lanes 0, 3 and 5 represent proteins present in total extracts for cells transformed with pQE60/KPf, before induction, 3 and 5 hours after induction with IPTG, respectively. The lane denoted by (+) represents purified PfHsp70 protein. The arrow on the right hand side represents mobility of a 70 kDa species. Molecular weight markers (in kDa) are shown in the extreme left hand side lane.

The exogenous production of DnaK and its variant, DnaK-V386L was confirmed by Western analysis using anti-*E. coli* DnaK antibodies (Figure 3.9B). As was the case with the *E. coli* Δ*dnaK52* cells, a lower amount of DnaK-V386L protein was produced in *E. coli dnaK103* cells than DnaK (wild type) protein. Despite this, both proteins were able to reverse thermosensitivity of the heat sensitive *E. coli dnaK103* cells to the same extent (Figure 3.9A).

Cells transformed with pQE30/PfHsp70, pQE30/PfHsp70-V401L, pQE30/PfHsp70-Q402K, pQE30/PfK and pQE60/KPf were unable to grow at 40°C. There was no evidence for the production of PfHsp70, PfHsp70-Q402K and PfK proteins by Western analysis. Based on SDS-PAGE analysis, the production of the chimera KPf was not confirmed (Figure 3.9B). Therefore, the fact that cells harbouring plasmids encoding these proteins failed to grow could be due to their failure to produce these proteins. However, PfHsp70-V401L is produced as a full length protein based on Western analysis (Figure 3.9A). Therefore, PfHsp70-V401L is unable to reverse the thermosensitivity of *E. coli* Δ*dnaK52* cells perhaps because of a functional deficiency since it is produced in detectable levels in these cells.

3.3.6.3 Complementation assay in *E. coli dnaK756* strain

At the permissive temperature of 37°C, all the *E. coli dnaK756* cells transformed with the different plasmids were able to grow (Figure 3. 10A). This shows that the presence of these plasmids in the cells did not inhibit cell growth. However, at the non-permissive temperature of 43.5°C, cells transformed with pQE30 and pQE60 plasmid vectors (negative control) were unable to survive heat stress. Cells transformed with pQE60/DnaK (positive control) and pQE60/DnaK-V386L were able to grow at 43.5°C. The production of DnaK and DnaK-V386L proteins was confirmed by Western analysis using anti-*E. coli* DnaK antibodies (Figure 3.10A). As observed before for the *E. coli* Δ*dnaK52* cells (Figure 3.8A) and *E. coli dnaK103* cells (Figure 3.9A), there is less DnaK-V386L protein produced than DnaK (wild type) protein in *E. coli dnaK756* cells (Figure 3.10A). The endogenous DnaK756 protein present in the *E. coli dnaK756* cells was detected using anti-*E. coli* DnaK antibodies and is represented by the protein band that was observed in *E. coli dnaK756* cells transformed with the pQE60 plasmid vector, probed with anti-*E. coli* DnaK antibodies (Figure 3.10A). The absence of a band observed for the anti-His based Western analysis conducted on *E. coli dnaK756* cells transformed with pQE30 (negative control) confirms that the anti-His

antibodies used were specific since they did not bind to endogenously expressed *E. coli* proteins (Figure 3.10A).

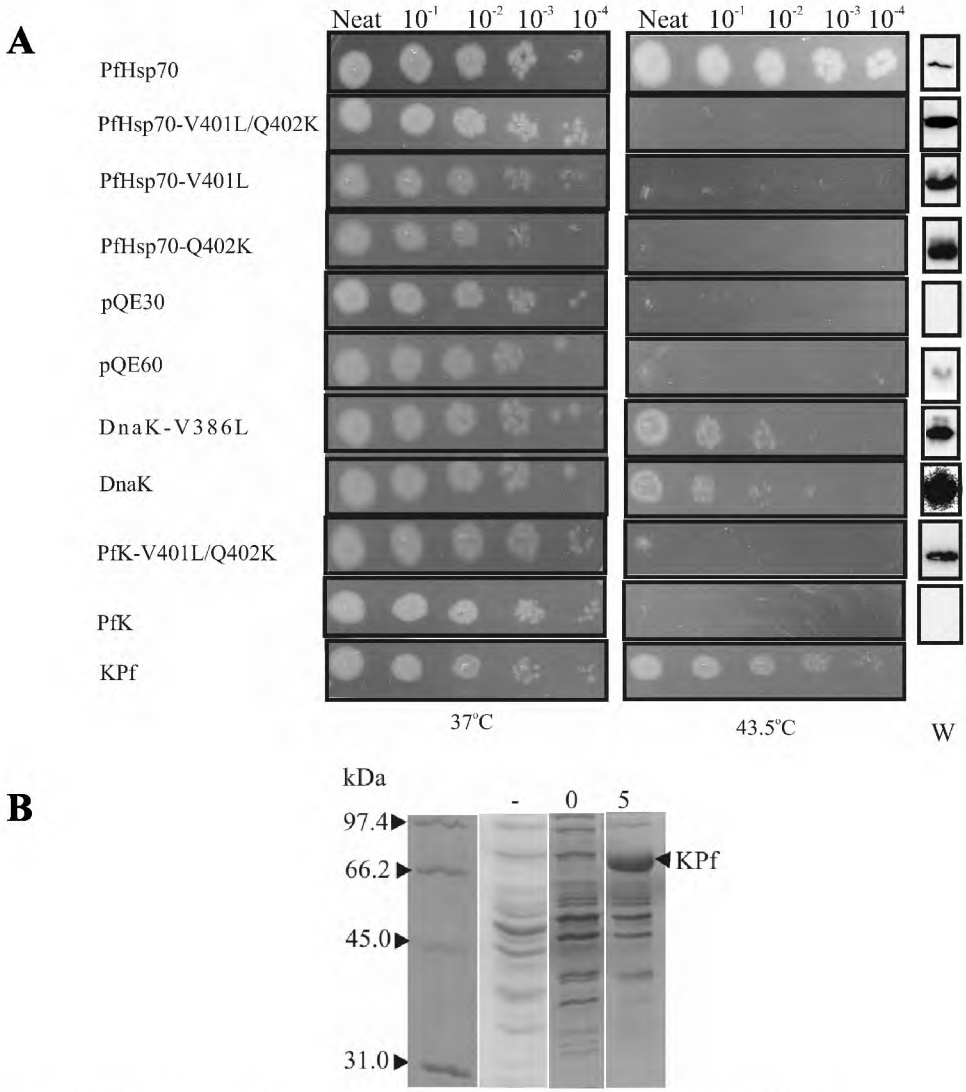

Figure 3.10 PfHsp70 was able to reverse thermosensitivity in *E. coli dnaK756* strain
(A) *E. coli dnaK756* cells transformed with plasmids pQE30/PfHsp70, pQE30/PfHsp70-V401L/Q402K, pQE30/PfHsp70-V401L, pQE30/PfHsp70-Q402K, pQE30 (negative control), pQE30/PfK-V401L/Q402K, pQE30/PfK, pQE60/KPf, pQE60/DnaK, pQE60/DnaK-V386L and pQE60 (negative control) were incubated at the permissive and non-permissive temperature of 37°C of 43.5°C, respectively. Western analysis for the production of PfHsp70 and its derivatives was conducted using anti-His antibodies, whilst the detection of DnaK and DnaK-V386L was carried out using anti-*E. coli* DnaK antibodies. The letter 'W' represents panels for the data from Western analysis. **(B)** Coomasie brilliant blue stained SDS-PAGE analysis for detection of KPf protein in *E. coli dnaK756* cells. The lane denoted by (-) represents proteins present in total extract for cells transformed with pQE60 plasmid. Lanes 0 and 5 represent proteins present in total extracts for cells transformed with pQE60/KPf, before induction, and 5 hours after induction with IPTG, respectively. The arrow on the right hand side shows the protein band representing KPf protein. Molecular weight markers (in kDa) are shown in the extreme left hand side lane.

Cells transformed with pQE30/PfHsp70 and pQE60/KPf were able to grow at 43.5°C (Figure 3.10A). The production of PfHsp70 was confirmed by Western analysis (Figure 3.10A). The SDS-PAGE analysis (Figure 3.10B) suggests that chimera KPf is produced by the *E. coli dnaK756* cells upon induction with IPTG. Therefore this strain of *E. coli* whose endogenous DnaK is suboptimally functional was able to support the expression of both PfHsp70 and KPf, leading to the reversal of the thermosensitivity of the *E. coli dnaK756* cells. On the other hand, the PfHsp70 derivatives - PfHsp70-V401L/Q402K, PfHsp70-V401L and PfHsp70-Q402K were unable to reverse the thermosensitivity of the *E. coli dnaK756* cells. The production of these proteins was demonstrated using Western analysis (Figure 3.10A). The fact that PfHsp70-V401L/Q402K, PfHsp70-V401L and PfHsp70-Q402K were produced in *E. coli dnaK756* cells despite their failure to reverse the thermosensitivity of these cells, suggests that these proteins had some functional deficiencies. In fact, data from Western analysis suggest that these proteins were produced at higher concentrations than PfHsp70 (Figure 3.10A). PfK-V401L/Q402K was produced as evidenced by anti-His antibody Western analysis, but was not able to suppress the thermosensitivity of the *E. coli dnaK756* cells (Figure 3.10A). Therefore, this protein could have failed to complement perhaps because of the linker substitutions that it harboured. The PfK chimeric protein (which had these mutations reversed), was not able to reverse the thermosensitivity of *E. coli dnaK756* cells either, and its production in these cells was not detected by Western analysis (Figure 3.10A). Thus, failure to reverse the thermosensitivity of *E. coli dnaK756* cells by this protein could be because it was not produced to sufficient levels.

3. 4 Discussion

This study analysed the *in vivo* chaperone function of PfHsp70 using an *E. coli* heterologous complementation system. Findings from this study indicate that PfHsp70, and its chimeric derivative, KPf, were able to reverse thermosensitivity in the *E. coli dnaK756* strain but not in *E. coli* strains that were completely devoid of DnaK function. The fact that PfHsp70 and KPf were able to complement for compromised DnaK function in *E. coli dnaK756* cells, suggests that the peptide binding domain of PfHsp70 can bind to a relatively broad range of protein substrates of eukaryotic and prokaryotic origins. Furthermore, PfHsp70 may have sufficient capacity to interact with misfolded *E. coli* protein substrates under thermal stress conditions, providing some level of cytoprotection to the cellular protein machinery.

Substrate specificity for Hsp70 proteins may be determined to some extent by their peptide binding domains with the β-sandwich region playing a key role in determining this specificity (Wu and Wang, 1999). However, some workers advocate that peptide binding specificity by the peptide binding domain is not the most important aspect that determines an Hsp70's function *in vivo*, but rather that functional specificity is determined by the physical interactions between Hsp70 domains and their regulatory co-chaperones, such as Hsp40 (James *et al.*, 1997; Lopez-Buesa *et al.*, 1998; Suh *et al.*, 1999; Tutar *et al.*, 2006). Therefore the ability of PfHsp70 to complement for DnaK function could be extrapolated to mean that PfHsp70 may have the capacity to interact sufficiently with the co-chaperone machinery of the *E. coli* cell, such that it can receive polypeptide substrates from these co-chaperones (such as DnaJ), thereby functionally integrating into the protein folding pathways of *E. coli.*

Because PfHsp70 reversed thermosensitivity in the *E. coli dnaK756* strain only, it is important to account for this observation. Furthermore, it is necessary to analyse whether PfHsp70 acted as a typical Hsp70 chaperone or that it might have merely interacted with DnaK756 to boost the latter's ability to function, for example by increasing its affinity for GrpE. A close study of the *dnaK756* allele could help address this issue. DnaK is essential for *E. coli* β-Galactosidase α-complementation (Ferreira and Alix, 2002). In the *E. coli dnaK756* system, α-complementation occurs when the strain is grown at 30°C but not at 37 or 40°C because the DnaK756 protein is inactive at the latter temperatures (Ferreira and Alix, 2002). In addition, in another study it was confirmed that the DnaK756 protein denatures at temperatures between 35°C and 40°C (Banecki *et al.*, 1992). The failure of expression of PfHsp70 in *E coli* Δ*dnaK52* and *dnaK103* strains could be due to a lack of a folding pathway for PfHsp70 in the absence of DnaK. Previous work on the production of a eukaryotic protein, $NADP^+$ reductase, showed that misfolding of the protein was enhanced in the *E. coli* Δ*dnaK52* strain compared to the *dnaK756* strain (Dionisi *et al.*, 1998). The results obtained in the present study supports this observation. For example, PfHsp70 and PfHsp70-V401L/Q402K whose production in *E coli* Δ*dnaK52* cells could not be confirmed by Western analysis (Figure 3.8A), were both expressed in *E. coli dnaK756* cells (Figure 3.10A).

Based on the aforesaid, it is conceivable that at the permissive temperature of 37°C (used to grow the cells prior to the plating stage), the DnaK756, supported by normal DnaJ levels in

the *E. coli dnaK756* strain, was able to facilitate the folding of PfHsp70 and KPf before its activity was abrogated at 43.5°C. The shift to 43.5°C, could have denatured the DnaK756 protein completely thus leaving PfHsp70 and KPf to functionally replace DnaK. The use of the *E. coli dnaK756* strain as a standard system in carrying out complementation assays with DnaK homologues under conditions of heat stress has been applied by others (Nimura *et al.*, 2001; Ferreira and Alix, 2002) and the reliability of this strain for these studies can only be based on the fact that the DnaK756 protein is compromised at high temperatures. Furthermore, the failure of the two PfHsp70 derivatives (PfHsp70-V401L and PfHsp70-Q402K) to functionally suppress thermosensitivity *in vivo* suggests that PfHsp70's ability to suppress the thermosensitivity of *E. coli dnaK756* cells was a specific chaperone function, and not a random event. This is especially illustrated by PfHsp70-V401L which could not reverse the thermosensitivity of *E. coli dnaK103* and *dnaK756* despite its expression in both strains.

Interdomain communication also plays an important part in the chaperone role of Hsp70 proteins with events that occur in the peptide binding domain known to influence conformational changes in the ATPase domain (Slepenkov and Witt, 2003). The linker region of DnaK that extends from residue number 385-393 (section 2.3.1) is a highly conserved region and mutations in this area of the protein were found to interfere with the chaperone role of the DnaK/DnaJ/GrpE system (Han and Christen, 2001). The conservative substitution (V386L) in the linker region of DnaK did not affect its *in vivo* function, whilst a double substitution (V401L/Q402K) and single substitutions (V401L and Q402K) in the linker region of PfHsp70 had deleterious effects on its *in vivo* function (Figure 3.10A). Interestingly, the V386L change resulted in reduced expression of DnaK (Figures, 3.8, 3.9 and 3.10) without affecting its ability to reverse thermosensitivity of the *E. coli* DnaK compromised strain. On the other hand, PfHsp70-V401L though produced in both *E. coli dnaK103* (Figure 3.9A) and *E. coli dnaK756* (Figure 3.10A) strains as a full length protein, failed to reverse the thermosensitivity of these cells (Figures 3.9A and 3.10A). Therefore, the V401L change favoured production of this form of PfHsp70 protein even in a strain that is completely devoid of DnaK function (*E. coli dnaK103*). Residues V401 and Q402 in PfHsp70 must be important for interdomain communication in PfHsp70 since they are located in the linker region, a segment known to be essential in Hsp70s. Besides its role in interdomain communication, the linker region is important for interaction of Hsp70 with

Hsp40 (Laufen *et al.*, 1999). Therefore, the mutations introduced in the linker segment of PfHsp70 (V401L and Q402K) may have abrogated its possible stimulation by *E. coli* DnaJ.

Using chimeric proteins, the possibility of allosteric communication between the functional segments (ATPase and peptide binding domains) of PfHsp70 and DnaK was investigated. *E. coli dnaK756* cells producing KPf were able to grow at non-permissive temperatures, whilst both PfK-V401L/Q402K and PfK could not reverse the thermosensitivity of these cells. PfK-V401L/Q402K production in *E. coli dnaK756* was confirmed by Western analysis (Figure 3.10A), hence its inability to complement must have been due to functional deficiency. On the other hand, PfK, (with reversed linker mutations) was not detected by Western analysis. Therefore, based on this assay, it is not clear whether PfK did not complement because of its structural-functional shortcomings or due to lack of production. Both *E. coli* Δ*dnaK52* and *E. coli dnaK103* strains could not express any of the chimeric proteins, and cells transformed with the plasmids encoding these proteins were sensitive to heat stress (Figures 3.8 and 3.9).

The fact that the KPf displayed *in vivo* function in *E. coli dnaK756* (Figure 3.10A) provides evidence that interdomain communication between the ATPase domain of DnaK and the peptide binding domain of PfHsp70 was possible. This observation contradicts that of Suppini *et al.* (2004), who found that a particular chimeric protein consisting of the ATPase domain of DnaK and the peptide binding domain of rat Hsc70 was unable to complement an *E. coli dnaK* mutant strain. The flexibility of interdomain interaction in chimeric Hsp70s has been confirmed by others (James *et al.*, 1997; Tutar *et al.*, 2006; this study). Although interdomain interaction of Hsp70s through the linker region is conserved, it is possible that the exceptional failure of this event, rather than lack of substrate recognition could explain why some chimeric Hsp70s fail to complement in *E. coli* DnaK mutant strains.

The fact that the V386L change in *E. coli* DnaK reduced the expression of this protein without affecting the protein's function *in vivo* highlights an interesting observation. It has been established that at high temperatures, DnaK relies not merely on improved expression to cope with its chaperone duties but that its chaperone activity increases as well (Sherman and Goldberg, 1993; McCarty and Walker, 1991). Therefore, one explanation of this could be that at the non-permissive temperatures used in this study DnaK-V386L was able to increase its chaperone activity to match that of DnaK. Further support for this hypothesis comes from the fact that some substitutions on DnaK tend to increase its ATPase and chaperone activities

by several magnitudes (Kamath-Loeb *et al.*, 1995; Buchberger *et al.*, 1999; Montgomery *et al.*, 1999).

The overproduction of DnaK in *E. coli* cells is toxic (Blum *et al.*, 1992; Sbai and Alix, 1998), and results obtained here show that the *E. coli* strains used were affected by DnaK toxicity, particularly *E. coli* Δ*dnaK52* and *E. coli dnaK756* strains. More severe DnaK toxicity was observed in *E. coli* Δ*dnaK52* cells (Figure 3.8A) than in *E. coli dnaK103* cells (Figure 3.9A), possibly because the higher DnaJ levels in *E. coli dnaK103* cells may have titrated out some of the DnaK thereby reducing its toxicity. The titration of Hsp70 protein by Hsp40 and misfolded proteins is a well known phenomenon (Beckman *et al.*, 1990; Tomoyasu *et al.*, 1998). Although DnaJ levels in *E. coli dnaK756* cells are normal, DnaK toxicity was severe in this strain possibly because of the combined action of the DnaK that was exogenously produced and the endogenously expressed DnaK756 protein.

In conclusion, this study showed that PfHsp70 and the chimeric protein, KPf, enabled an *E. coli dnaK* mutant strain to grow under thermal stress conditions. Previous studies have shown that PfHsp70 displays *in vitro* chaperone features such as a relatively high basal ATPase activity (Matambo *et al.*, 2004), and that the protein was associated with PfHsp90 in potentially functional units (Banumathy *et al.*, 2003). Although the role of PfHsp70 in the parasite is still largely unclear, there is now substantial evidence that PfHsp70 could function as a molecular chaperone in the *P. falciparum* life-cycle. Chapter 4 covers a report on the characterisation of key residues in the peptide binding domain of PfHsp70.

Chapter Four

Analysis of the PfHsp70 substrate binding cavity

4.1 Introduction

Hsp70 interacts with the peptide substrate through hydrogen bonds, Van der Waal forces, and hydrophobic contacts (Zhu *et al.*, 1996; Rüdiger *et al.*, 1997). Three regions have been identified as crucial in the interaction of DnaK with its substrate; the α-helical lid, the arch formed by residues A429 and M404 and the hydrophobic central pocket that is defined by the V436 residue which regulates substrate binding affinity (Figure 4.1; Mayer *et al.*, 2000b). In this study the structure that is formed by these three elements will be referred to as the substrate binding cavity.

The introduction of a V436F substitution in the hydrophobic pocket of DnaK reduced its affinity for peptide substrate without affecting its specificity for substrates (Rüdiger *et al.*, 2000). The preservation of substrate specificity in the DnaK-V436F mutant shows that the hydrophobic pocket is capable of interacting flexibly with a range of substrates (Rüdiger *et al.*, 2000). However, despite this, the V436F change in DnaK had fatal consequences due to loss of substrate affinity emanating from the introduction of the large side chain of the phenylalanine residue (Mayer *et al.*, 2000b). The V436 hydrophobic residue of DnaK is the only residue in the substrate binding cavity of DnaK whose side chain seems to interact with the central leucine residue on the bound peptide (Gragerov *et al.*, 1994; Zhu *et al.*, 1996). This leucine residue (of the peptide substrate) is buried deep in the substrate binding cavity of DnaK (Zhu *et al.*, 1996).

Whilst, the M404A change in DnaK did not result in detectable change in affinity for substrates *in vitro*, introduction of mutations A429W, M404A/A429W in the hydrophobic arch of DnaK reduced its ability to bind substrates *in vitro* (Rüdiger *et al.*, 2000). The DnaK variants (DnaK-A429W and DnaK-M404A/A429W) had compromised *in vivo* function compared to wild type protein (Mayer *et al.*, 2000b). Based on complementation assays, the DnaK-M404A did not show functional deficiencies compared to that of wild type protein (Mayer *et al.*, 2000b). However, only upon exposing the *E. coli* Δ*dnaK52* cells to high IPTG

concentration to maximise the production of the DnaK-A429W protein, was the partial *in vivo* function detected (Mayer *et al.*, 2000b).

The universal nature of the arch and hydrophobic pocket residues of Hsp70s has been confirmed by a recent study showing the importance of these residues in another *E. coli* Hsp70 homologue, HscA (Tapley *et al.*, 2006). HscA has residues M401/F426 (Tapley *et al.*, 2006) corresponding to M404/A429 arch residues in DnaK (Zhu *et al.*, 1996; Rüdiger *et al.*, 2000). Interestingly, whilst an A429W mutation in DnaK reduced its affinity and specificity for a section of DnaK substrates (Rüdiger *et al.*, 2000); an equivalent substitution in HscA, F426A, did not alter substrate specificity (Tapley *et al.*, 2006). This is in spite of the fact that F426 is the only residue whose β-carbon makes contact with the peptide substrate (Tapley *et al.*, 2006). This indicates that substrate specificity in Hsp70s is not restricted to one residue but could be regulated by several residues (Tapley *et al.*, 2006).

HscA recognises different substrates from *E. coli* DnaK, and prefers peptides with a central proline residue (Hoff *et al.*, 2003) as opposed to DnaK which favours the presence of a leucine residue in the same position (Rüdiger *et al.*, 1997). The M433V mutation completely changed the substrate specificity of HscA, leading to its preference for residues with leucine in the central position (Tapley *et al.*, 2006). Thus, the HscA hydrophobic pocket residue is not only important in regulating substrate affinity, but influences specificity as well (Tapley *et al.*, 2006). As opposed to this, the DnaK-V436F mutant only lost its affinity for substrates but retained its substrate specificity (Rüdiger *et al.*, 2000). Therefore, this suggests different roles for hydrophobic pocket residues of HscA and DnaK. HscA and its yeast homologue, Ssq1, share the same substrate recognition motif (LPPVK), despite the fact that Ssq1 has a V472 in its hydrophobic pocket as opposed to the M433 in HscA (Hoff *et al.*, 2002; Dutkiewicz *et al.*, 2004; Tapley *et al.*, 2006). This further demonstrates that Hsp70's specificity for substrates must be regulated through several residues in its peptide binding domain.

It has been traditionally accepted that in the ATP bound state Hsp70 has low affinity for substrate which increases in the ADP bound state (Suh *et al.*, 1999), with the lid segment closing over the β-sheet to stabilise peptide binding (Fernandez-Sáiz *et al.*, 2006). However, the fact that the substrate is brought to an Hsp70 protein in the ATP bound state has led to interest in comparing the binding kinetics of both the ATP and ADP bound states of Hsp70

(Mayer *et al.*, 2000b). A correlation that was observed in the K_d (dissociation constant) values of the ATP and ADP bound states, led to the conclusion that substrates enter and leave the Hsp70 substrate binding cavity in both the ATP and ADP bound states (Mayer *et al.*, 2000b). The only difference is that in the ATP bound state, the frequency of transition between the open and closed transition state of the substrate binding cavity is higher than in the ADP bound state (Mayer *et al.*, 2000b).

It has previously been proposed that the α-helical lid plays an important role in stabilising the β-sandwich through its interaction with loops of the β-sandwich (Moro *et al.*, 2004). However, findings by Pellecchia *et al.* (2000) contradict this observation. Removal of the lid of DnaK did not change the orientation of loops $L_{1,2}$ and $L_{4,5}$ whose role is to stabilise the substrate binding cavity (Figure 4.1; Pellecchia *et al.*, 2000). This is despite the fact that the lid has direct contact with these loops (Fernandez-Sáiz *et al.*, 2006). Residues M404 (Figure 4.1), L441 and A448 in loop $L_{4,5}$ are thought to form hydrophobic associations that stabilise these loops (consequently stabilising the substrate binding cavity), even in the lidless form of DnaK (Pellecchia *et al.*, 2000). This suggests a less important role for the lid in maintaining the stability of the substrate binding cavity (Pellecchia *et al.*, 2000). It has been further hypothesised that loops $L_{3,4}$ (Figure 4.1) also regulate substrate binding directly, by acting as 'porters' of the substrate binding cavity (Pellecchia *et al.*, 2000).

The interaction of Hsp70 with its substrate and co-chaperone partners induces both localised and global conformational changes (Zhang and Zuiderweg, 2004). Some residues that are important for peptide binding play an important role in the interdomain communication of Hsp70 (Ha *et al.*, 1997; Montgomery *et al.*, 1999). The E543 residue of bovine Hsc70 is implicated in both interdomain coupling and substrate binding through its contribution to the formation of a latch that holds the substrate in place (Ha *et al.*, 1997). In *E. coli* DnaK, residues that participate in both substrate binding and interdomain coupling have been identified (Montgomery *et al.*, 1999). In addition, some changes that affect Hsp70 peptide binding interfere with co-chaperone regulation, consequently impairing interdomain coupling (Laufen *et al.*, 1999; Montgomery *et al.*, 1999). An example of this is the *E. coli* DnaK-V436F change, which abrogates the stimulation of DnaK by DnaJ (Laufen *et al.*, 1999). Pellecchia *et al.* (2000) observed that peptide binding results in structural changes whose influence migrate from the peptide binding cleft to loops $L_{2,3}$ (Figure 4.1). Conversely, chemical shifts originating from loops $L_{2,3}$ have influence on the substrate binding cavity

(Pellecchia *et al.*, 2000). Indeed, a number of mutations in the substrate binding cavity of DnaK that affect its allosteric behaviour have been traced to be in loops $L_{2,3}$ (Buchberger *et al.*, 1995; Montgomery *et al.*, 1999).

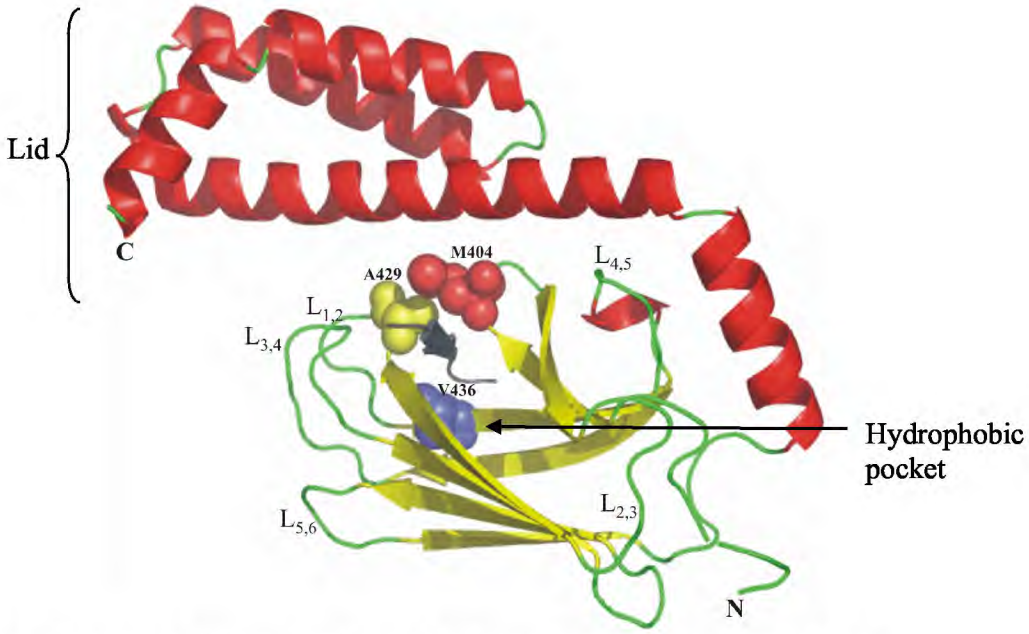

Figure 4.1 The interaction between the substrate binding cavity and loop structures of DnaK
Residues A429 (in yellow colour) and M404 (in red colour) form the arch that regulates peptide binding (Rüdiger *et al.*, 2000). Residue V436 (in blue colour) is positioned in the hydrophobic pocket of the substrate binding cavity. Loops ($L_{5,6}$, $L_{3,4}$, $L_{1,2}$, $L_{4,5}$, $L_{2,3}$) that are connected to the β-sandwich are shown. The hydrophobic pocket is defined by the β-sheets (yellow). The bound peptide (grey) is shown held in the substrate binding cavity. The N- and C-termini are shown as 'N' and 'C', respectively (1DKX.pdb; Zhu *et al.*, 1996). The figure was generated using PyMol (DeLano, 2002).

The fact that PfHsp70 can complement compromised DnaK function in *E. coli* suggests that it recognises at least a section of *E. coli* substrates *in vivo* (section 3.3.6.3; Shonhai *et al.*, 2005). Substrate specificity of Hsp70 proteins may be determined to some extent by their peptide binding domains, with the β-sandwich region playing a key role in determining this specificity (Wu and Wang, 1999). PfHsp70, possess an 'inverted' arch, characteristic of most eukaryotic Hsp70s (made up of residues A404/Y429) as opposed to that of DnaK (made up of residues M404/A429) (Rüdiger *et al.*, 2000). This configuration of the arch of eukaryotic Hsp70s is thought to enable them to meet the demands of their protein folding pathways (Mayer and Bukau, 2005).

This chapter is a report on studies that were carried out to analyse the role of residues that define the arch and the hydrophobic pocket of PfHsp70. Using site-directed mutagenesis,

changes were made to residues that define the putative arch and hydrophobic pocket of PfHsp70. The same mutations were also introduced onto the chimera KPf. This chimera, like PfHsp70, was able to reverse thermosensitivity of the *E. coli dnaK756* strain (section 3.3.6.3). The derivative proteins were analysed for their ability to complement for DnaK function using the *E. coli dnaK756* strain.

The following is a summary of the specific objectives of this study:

1. Investigating the role of residues A419 and Y444 that potentially define the putative hydrophobic arch of PfHsp70.
2. Examining whether a switch of the positions of residues A419 and Y444 has the same compensatory effect on the function of PfHsp70 as observed for *E. coli* DnaK (Rüdiger *et al.*, 2000; Mayer *et al.*, 2000b).
3. Probing the role of residue V451 that occurs in the putative hydrophobic pocket of PfHsp70.
4. Analysing the effect of the changes made on the arch and hydrophobic pocket residues on PfHsp70 (wild type protein) relative to the same mutations introduced on its derivative chimera KPf.

4.2 Experimental procedures

4.2.1 Materials

The plasmids and the *E. coli dnaK756* strain used in this study have been previously discussed (section 3.2). Growth conditions applied in this study for the *E. coli dnaK756* cells were the same as previously described (Table 3.1; section 3.2.4).

4.2.2 Rationale used to introduce arch and hydrophobic pocket substitutions in PfHsp70

Based on previous studies conducted on the *E. coli* DnaK substrate binding cavity (Mayer *et al.*, 2000b), a similar strategy was used to investigate the role of key residues for the substrate binding cavity of PfHsp70 (Figures 2.6 and 4.2). The objective of the study was to investigate the contribution of arch residues (A419 and Y444) of PfHsp70 and its hydrophobic pocket defined by the V451 residue (Figure 4.2). In order to elucidate the role of the arch and hydrophobic pocket residues of the substrate binding cavity of PfHsp70,

mutations were conducted in this subdomain in both PfHsp70 and KPf proteins based on the approach employed by Mayer *et al.* (2000b).

4.2.3 Generation of modified plasmids

Plasmids pQE30/PfHsp70 and pQE60/KPf were used as parental DNA to generate modified plasmids encoding PfHsp70 and KPf with mutations in the substrate binding cavities. Site-directed mutagenesis was carried out using the primers provided (Table B.2; Appendix B). The Stratagene (U.S.A) QuikChange site-directed mutagenesis kit was used to modify the plasmids, following the instructions of the supplier (Appendix A.6). The following modified derivatives were generated from pQE30/PfHsp70: pQE30/PfHsp70-A419Y (encoding for PfHsp70-A419 protein), pQE30/PfHsp70-Y444A (encoding for PfHsp70-Y444A protein), and pQE30/PfHsp70-V451F (encoding for PfHsp70-V451F protein). The modified plasmid pQE30/PfHsp70-A419Y was used as template for the generation of plasmid pQE30/PfHsp70-A419Y/Y444A (encoding for PfHsp70- A419Y/Y444A). The same primers were used to introduce similar changes in the pQE60/KPf plasmid resulting in the following modified plasmids: pQE60/KPf-A404Y (encoding for KPf-A404Y protein), pQE60/KPf-Y429A (encoding for KPf-Y429A protein), and pQE60/KPf-A436F (encoding for KPf-A436F protein). The pQE60/KPf-A404Y plasmid was used as the template to generate pQE60/KPf-A404Y/Y429A (encoding for KPf-A404Y/Y429A protein). All the changes made were verified through DNA sequencing.

4.2.4 Complementation assays

E. coli dnaK756 cells were transformed with plasmids encoding the proteins, PfHsp70 and KPf, as well as their respective derivatives with mutations in the substrate binding cavities. The plasmid pQE30/PfHsp70-V401L/Q402K, encoding PfHsp70-V401L/Q402K protein (with abrogated *in vivo* function, section 3.3.6.3) was used as a negative control. A second negative control used in this study was pQE60 plasmid vector, since this vector was used to construct the plasmids encoding KPf and DnaK. The pQE60/DnaK plasmid that encoded *E. coli* DnaK was used to transform *E. coli dnaK756* cells as a positive control. To assess functional capabilities of the proteins, complementation assays were conducted using a previously described procedure (section 3.2.4).

4.2.5 Protein expression studies

In order to verify whether the *E. coli dnaK756* cells successfully produced PfHsp70 and KPf as well as their respective derivatives, protein expression studies were conducted by previously used methods (section 3.2.5). For SDS-PAGE analysis, 15 µl of cell lysate was loaded in each lane of the gel. The primary antibody used for Western analysis (Appendix A.9 and A.10) was diluted to a concentration of 1: 15 000 for either anti-*E. coli* DnaK or anti-His antibodies, whilst the concentration of secondary antibody used was 1 : 10 000.

4.3 Results

4.3.1 The architectural organisation of arch and hydrophobic pocket of PfHsp70 and its variants

PfHsp70 possesses an 'inverted' arch characteristic of eukaryotic Hsp70s and a conserved hydrophobic pocket (section 2.3.2.4; Rüdiger *et al.*, 2000). Using the *E. coli dnaK756* strain as the host for complementation assays, the arch and hydrophobic pocket residues in the of PfHsp70 were mutated and their functional contribution analysed. Since two Hsp70 proteins carrying the peptide binding domain of PfHsp70 (PfHsp70 and chimera KPf) were available, the study was carried out using the two proteins. The analysis of the arch and hydrophobic pocket substitutions in both KPf and PfHsp70 proteins was conducted in order to establish the role of these structures relative to the contribution of the different ATPase domains of these proteins.

The organisation of the arch and hydrophobic pocket residues of PfHsp70 and its derivatives carrying amino acid substitutions are shown (Figure 4.2). The substrate binding cavity of PfHsp70 consists of the arch (made up of residues A419 and Y444) and a hydrophobic pocket defined by the V451 residue (Figure 4.2). In order to elucidate the role of the arch and hydrophobic pocket residues of the substrate binding cavity of PfHsp70, mutations were introduced in this subdomain in both PfHsp70 and KPf proteins based on the approach employed by Mayer *et al.* (2000b) (Figure 4.2). The Y444A change is predictably supposed to produce a widened gap on the arch, which favours entry and exit of substrates. The A419Y change is expected to restrict access to the arch by substrate. The A419Y/Y444A double substitution was used to investigate whether switching the positions of these arch residues

(Figure 4.2) would lead to functional compensation. The role of the conserved hydrophobic pocket residue was investigated by the V451F change. This change led to the hydrophobic pocket being occupied by a much bigger residue that would be expected to lower affinity for the substrate.

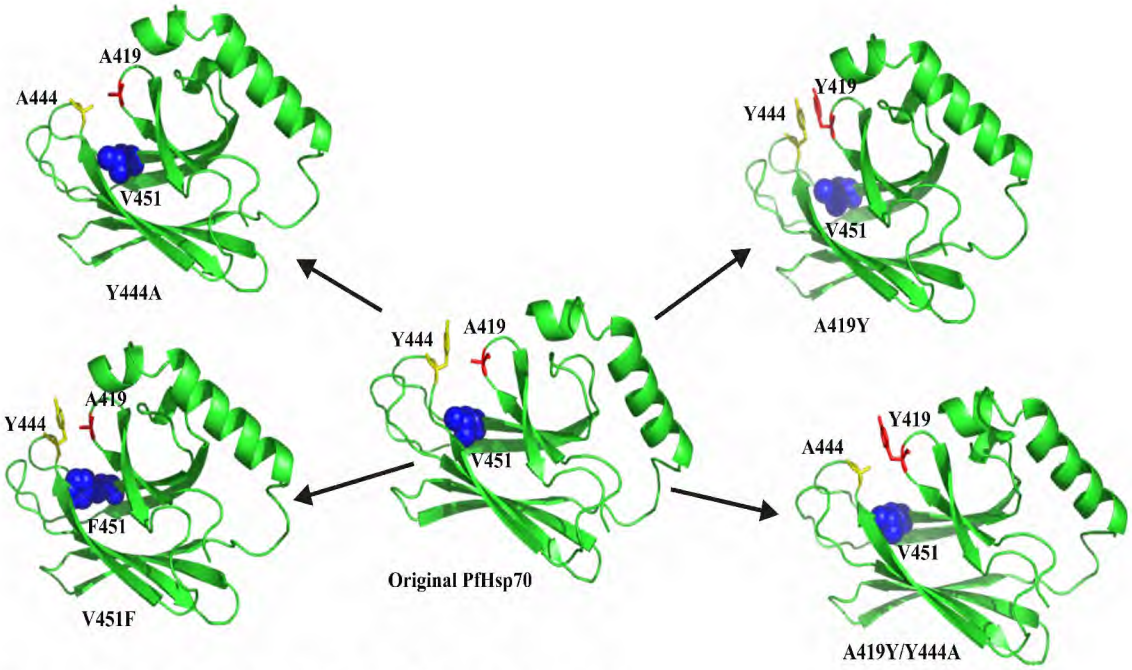

Figure 4.2 PfHsp70 arch and hydrophobic pocket mutants
Ribbon representations for a section of the PBD of PfHsp70 showing arch and hydrophobic pocket substitutions that were introduced in the substrate binding cavity of PfHsp70 (wild type protein), and its chimeric derivative, KPf. The positions of residues shown here are reflective of the locations of the residues in full length PfHsp70 protein. Residues highlighted in stick form constitute the arch, whilst the hydrophobic pocket residue is presented in sphere form. The homology model of PfHsp70 PBD was generated based on the crystal structure of Hsc70 (1YUW.pdb; Jiang *et al.,* 2005). The figure was visualised using PyMol (DeLano, 2002).

4.3.2 PfHsp70 arch derivatives displayed marginally compromised function

Plasmid constructs expressing PfHsp70 and its substrate binding cavity derivatives as well as control plasmids were used to transform *E. coli dnaK756* cells. At the permissive temperature of 37°C, all the *E. coli dnaK756* cells transformed with various plasmids were able to grow (Figure 4.3). As expected, cells transformed with pQE30/PfHsp70-V401L/Q402K and pQE60 (negative controls) were not able to grow at the non-permissive temperature of 43.5°C. This indicates that the *E. coli dnaK756* cells are heat sensitive at this temperature, confirming that the endogenous DnaK756 protein is unable to protect these cells from heat. Cells transformed with pQE60/DnaK were able to grow at the non-permissive temperature of 43.5°C, indicating that the exogenously expressed *E. coli* DnaK was

responsible for the reversal of the thermosensitivity. Cells expressing PfHsp70 were able to grow at 43.5°C as expected (section 3.3.6.3).

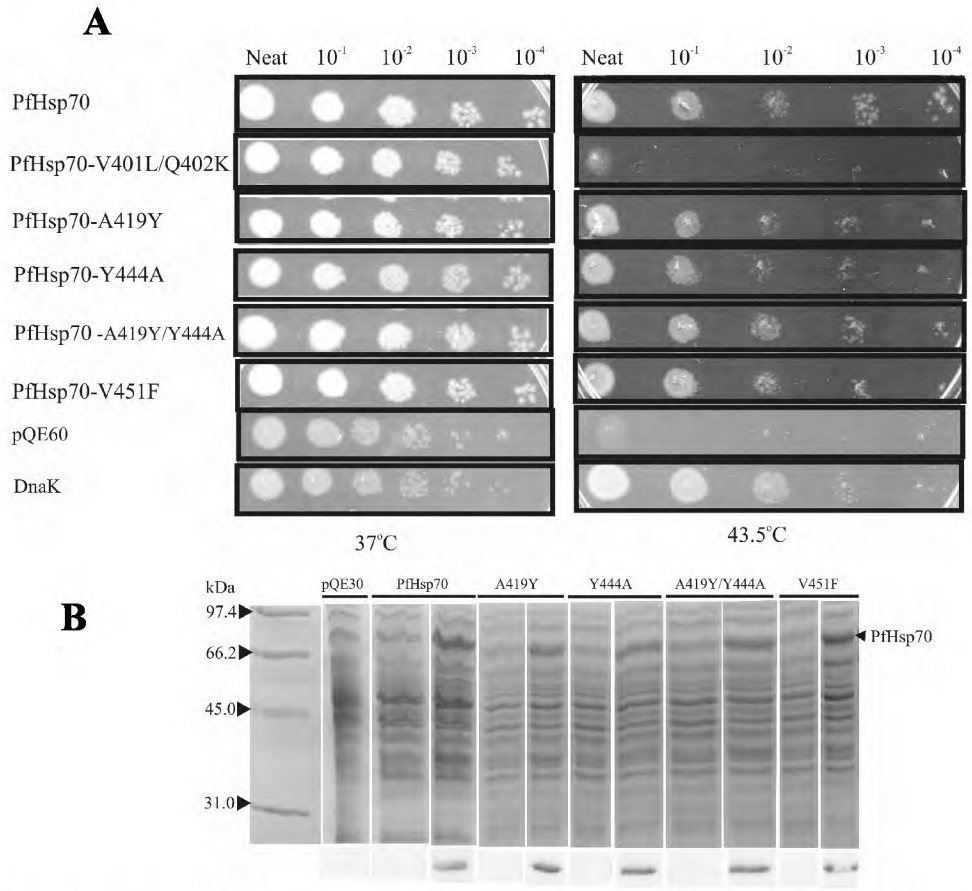

Figure 4.3 PfHsp70 arch and hydrophobic pocket mutants displayed marginal loss of *in vivo* function
(A) *E. coli dnaK756* cells transformed with plasmid constructs expressing PfHsp70 and its derivatives were incubated at 37°C and 43.5°C. At the non-permissive temperature of 43.5°C cells expressing all the PfHsp70 derivatives were slightly less resilient to heat stress compared to those producing the wild type protein. The negative control constituted cells transformed with plasmids pQE60 and pQE30/PfHsp70-V401L/Q402K. As positive control cells that had been transformed with pQE60/DnaK were used. **(B)**. SDS-PAGE (upper panels) and Western analysis (lower panels) for the exogenous expression of PfHsp70 and its substrate binding cavity derivatives in *E. coli dnaK756* cells. Cells transformed with plasmid vector (pQE30) were used as negative control. The labels on the top of the gel panels represent the different proteins that were analysed as well as the vector control (pQE30). In each case, the left and right hand side lanes represent the sample that was taken before induction and 5 hours after induction with IPTG, respectively. The arrow on the extreme right hand side represents mobility expected of a His-tagged PfHsp70 species. Molecular weight markers (in kDa) are shown in the extreme left hand side lane.

All the substrate binding cavity derivatives of PfHsp70 displayed a fractional deficiency in supporting growth of *E. coli dnaK756* cells at the non-permissive temperature used. Since all the PfHsp70 derivatives were equally produced based on Western analysis (Figure 4.3B), the marginal *in vivo* functional deficiency displayed by these proteins must have been due to the structural adjustments introduced in their substrate binding cavities. The lack of distinct phenotypic expressions observed for *E. coli dnaK756* cells expressing the mutated PfHsp70

proteins, perhaps indicates that the *in vivo* assay lacked the degree of resolution required to unmask the functional deficiencies of these proteins.

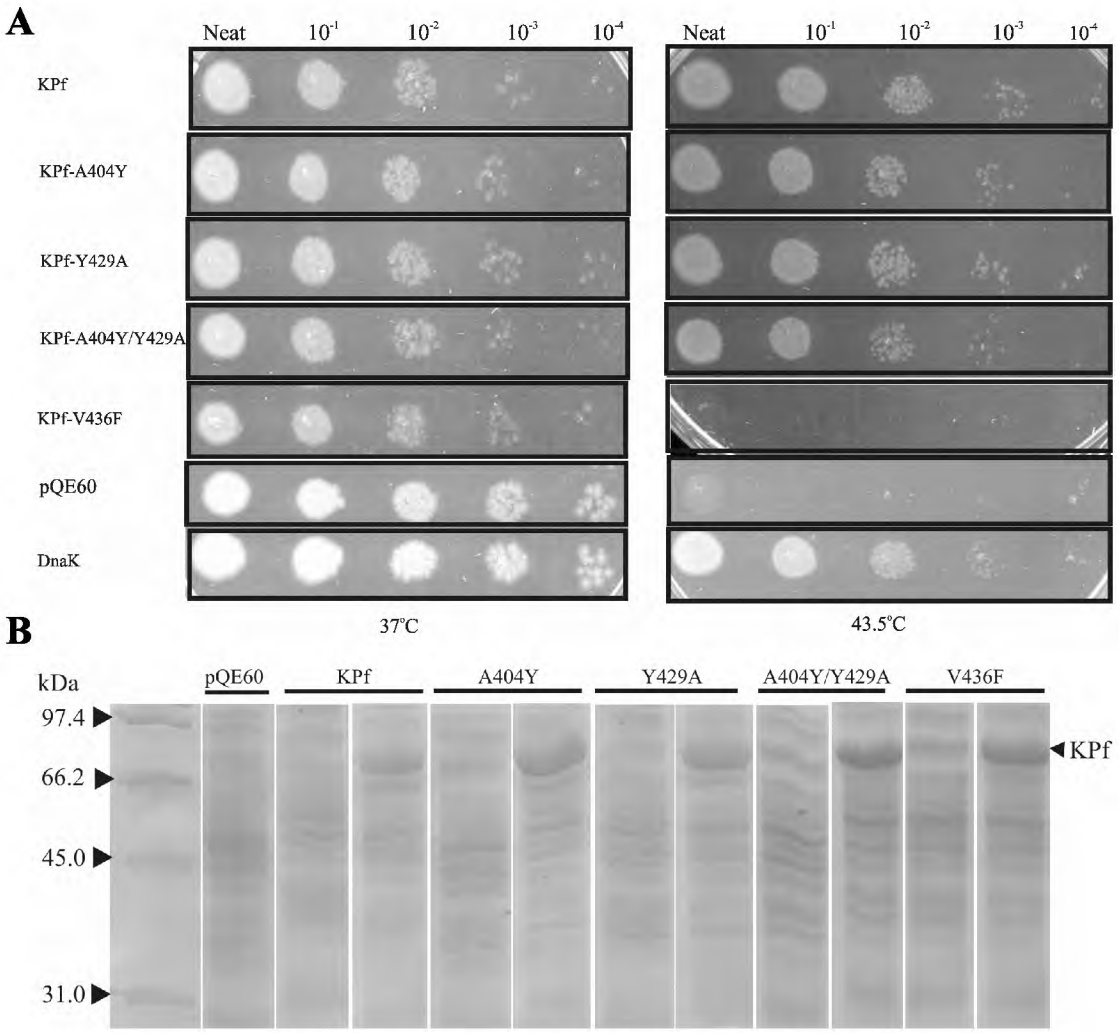

Figure 4.4 The hydrophobic pocket mutation abrogated the *in vivo* function of KPf
(A) *E. coli dnaK756* cells transformed with plasmid constructs expressing KPf and its substrate binding cavity mutants were incubated at 37°C and 43.5°C. At the non-permissive temperature (43.5°C) cells expressing KPf carrying the V436F mutation in the substrate binding cavity could not resist heat stress. However, all the other variants of KPf were able to resist heat stress at 43.5°C. The negative control consisted of cells transformed with pQE60 plasmid vector whilst the positive control was represented by cells transformed with the pQE60/DnaK plasmid. **(B)** SDS-PAGE analysis for the exogenous expression of chimera KPf and its substrate binding cavity derivatives in *E. coli dnaK756* cells. Cells transformed with plasmid vector (pQE60) were used as negative control. The labels on the top of the gel panels represent the different proteins that were expressed as well as the vector control (pQE60). In each case, the left and right hand side lanes represents the sample that was taken before induction and 5 hours after induction with IPTG, respectively. The arrow on the extreme right hand side represents mobility expected of the KPf chimera. Molecular weight markers (in kDa) are shown in the extreme left hand side lane.

4.3.3 Arch substitutions in KPf did not affect its *in vivo* function

Arch substitutions introduced in the substrate binding cavity of KPf (A404Y, Y429A and A404Y/Y429A) did not influence its *in vivo* function, based on complementation assays (Figure 4.4A). Judging by data from the SDS-PAGE analysis (Figure 4.4B), the mutant proteins were produced to the same level as the original KPf protein upon induction with IPTG. This negates the possibility that the complementation data may have been influenced by different protein levels in the cells. However, Western analysis would have been a more definitive way to estimate the levels of each protein in the system.

4.3.4 The V436F hydrophobic change in KPf abrogated its *in vivo* function

The V436F substitution introduced in the hydrophobic pocket of KPf chimera led to a complete loss of function *in vivo* at 43.5°C (Figure 4.4A). This is despite the fact that SDS-PAGE analysis showed that the protein was produced to a level comparable to the original KPf chimera (Figure 4.4B). Therefore, the V436F change must have been responsible for KPf's failure to suppress the thermosensitivity of *E. coli dnaK756* cells. This observation is interesting, since a similar substitution introduced on the full length PfHsp70 only led to a marginal functional deficiency *in vivo* (Figure 4.3A). However, in DnaK, a similar mutation led to abrogation of function based on a complementation study (Mayer *et al.*, 2000b).

4.4 Discussion

The role of the arch and hydrophobic pocket of Hsp70 proteins as important functional structures has received a lot of attention both in prokaryotes and eukaryotes (Zhu *et al.*, 1996; Mayer *et al.*, 2000a,b; Rüdiger *et al.*, 2000; Jones and Masison, 2003; Knieszner *et al.*, 2005; Tapley *et al.*, 2006). PfHsp70 possesses an 'inverted' arch characteristic of eukaryotic Hsc70 (section 2.3.2.4; Rüdiger *et al.*, 2000). The objective of this study was to establish the role of the arch and hydrophobic pocket residues of PfHsp70. All the PfHsp70 arch and hydrophobic pocket mutants revealed marginal functional compromise (Figure 4.3A). In the KPf background, except for the hydrophobic residue mutation, the rest of the derivatives carrying changes in the substrate binding cavity displayed functional capability comparable to wild type protein (Figure 4.4A). The V436F change in KPf was accompanied by a complete loss of function *in vivo*.

The change on the PfHsp70-Y444A/KPf-Y429A would be expected to introduce a widened arch structure and reduced hydrophobic interactions promoted by the original arch residues (Figure 4.2; Mayer *et al.*, 2000b). This promotes the movement of substrate in and out of the substrate binding cavity (Mayer *et al.*, 2000b). Based on *in vitro* assays, this mutation did not alter the binding specificity of a DnaK variant with the same change (Rüdiger *et al.*, 2000). In addition, an *in vivo* thermosensitivity-based complementation assay confirmed the DnaK variant had the same functional capability as wild type protein (Mayer *et al.*, 2000b). Therefore, the results for this mutation in the KPf background is congruent with findings obtained in the equivalent *E. coli* DnaK variant based on complementation assays (Mayer *et al.*, 2000b). Therefore, the marginal loss of function observed in PfHsp70 for the same mutation suggests that this could be due to the difference in affinity for *E. coli* substrates between PfHsp70, KPf and *E. coli* DnaK.

The PfHsp70-A419Y/KPf-A404Y mutation would be expected to increase potential hydrophobic forces and at the same time, introduce a large residue that tends to block the arch, as observed for similar changes in *E. coli* DnaK (Rüdiger *et al.*, 2000). The PfHsp70 derivative carrying this change only marginally failed to protect *E. coli dnaK756* cells against heat stress (Figure 4.3A). On the other hand, KPf-A404Y did not show evident loss of function *in vivo* (Figure 4.4A). The *in vivo* phenotype based on PfHsp70-A419Y affirms results that were obtained for a similar change in *E. coli* DnaK-A429W which also resulted in a marginal loss of function (Mayer *et al.*, 2000b). *In vitro* results obtained for the *E. coli* DnaK variant, showed that the protein had reduced affinity for a fraction of DnaK substrates (Rüdiger *et al.*, 2000). The DnaK-A429W mutant was able to recover affinity for some of its peptide substrates when the flanking acidic residues on the peptides were switched to basic residues (Rüdiger *et al.*, 2000). It was proposed therefore that the A429W change led to the introduction of a large side chain that disfavoured the binding of peptides with flanking acidic residues (Rüdiger *et al.*, 2000).

In DnaK, the affinity of the double mutant M404A/A429W for peptides was largely the same as for A429W alone (Rüdiger *et al.*, 2000). PfHsp70-A419Y/Y444A shows a marginal loss of function compared to wild type protein (Figure 4.3A). This double substitution introduces a similar phenotype to that obtained for PfHsp70-A419Y. In KPf, the same changes (A404Y/Y429A) led to a protein whose functional contribution *in vivo* was the same as that of wild type protein (Figure 4.4A). Thus in the PfHsp70 background, the double substitution,

though having a functional compensatory effect led, to a marginal loss of *in vivo* function. The phenotypic variation between the PfHsp70 and KPf backgrounds further suggests that the orientations of their substrate binding cavities are different. This could be as a result of the differential input of their ATPase domains to the architectural organisation of the substrate binding cavity. Alternatively, the contribution of co-chaperones that marshal these two Hsp70s through their different ATPase domains is responsible for their ability to display distinct functional features (James *et al.*, 1997). An *in vitro* analysis comparing the peptide binding affinities of PfHsp70 and KPf in the presence and absence of an Hsp40 co-chaperone could be useful towards drawing some insights in the mechanisms of action underscoring the differences in the behaviour of these proteins. Such a study could establish whether the binding affinities of these proteins vary because of co-chaperone input or differ in architectural orientation, regardless of the input from co-chaperones.

The V451F change in the hydrophobic pocket of PfHsp70, though largely expected to show the most drastic changes, only showed a marginal loss of function *in vivo* (Figure 4.3A). However, as observed in DnaK for a similar substitution, KPf-V436F displays a lethal phenotype in the *E. coli dnaK756* cells (Figure 4.4A). Based on SDS-PAGE data (Figure 4.4B), there is evidence for the expression of KPf-V436F protein and hence its failure to support growth of the thermosensitive *E. coli* cells must have been due a functional deficiency and not a lack of protein production. The abrogation of function of KPf-V436F variant is anticipated since the bulky phenylalanine residue introduced would be expected to block the hydrophobic pocket, a phenomenon that led to a lethal loss of function in an *E. coli* DnaK study (Mayer *et al.*, 2000b). The hydrophobic pocket residue is extensively conserved across the Hsp70 kingdom (Rüdiger *et al.*, 2000) and it is the only residue in the substrate binding cavity of *E. coli* DnaK whose side chain faces the central leucine residue of the peptide substrate (Gragerov *et al.*, 1994; Mayer *et al.*, 2000b).

The *E. coli* DnaK-V436F variant displayed reduced affinity for substrates without altering substrate specificity (Mayer *et al.*, 2000b; Rüdiger *et al.*, 2000). Therefore, the possible retention of substrate specificity by PfHsp70-V451F may have overshadowed the functional loss incurred through reduced affinity for substrates. The variation in behaviour between KPf-V436F and PfHsp70-V451F could be due to the differential contributions of their respective ATPase domains towards peptide binding kinetics. It is therefore likely that in the *E. coli* cell, KPf or KPf-V436F could have interacted with DnaJ and possibly with GrpE as

well. However, PfHsp70 or PfHsp70-V451F whose ability to interact with these co-factors in the *E. coli* cell could not be guaranteed, may have largely operated through the holdase function (Slepenkov and Witt, 2002; Popp *et al.*, 2005), thereby inhibiting protein aggregation. Therefore, the possible interaction of KPf-V436F with a full complement of co-chaperones and NEF's, may have altered the architecture of its hydrophobic pocket, further leading to loss of affinity for substrates. This is especially convincing given the fact that co-chaperones are believed to define functional capabilities of an Hsp70 protein more than the physical outline of the peptide binding domain (James *et al.*, 1997; Jones and Masison, 2003; Tapley *et al.*, 2006; Tutar *et al.*, 2006).

Some residues in the peptide binding domain of Hsp70 that are crucial for substrate binding, also play a role in the regulation of Hsp70 by Hsp40 co-chaperones (Ha *et al.*, 1997; Chang *et al.*, 2001; Tutar *et al.*, 2006). Coincidentally, the V436F substitution is known to interfere with interaction of DnaK with DnaJ (Laufen *et al.*, 1999; Mayer *et al.*, 2000b); confirming the intimacy of DnaJ regulation of DnaK and its effect on the dynamics of the hydrophobic pocket. To this end, there is speculation that the J domain of an Hsp40 interacts with the peptide binding domain of Hsp70, and is only displaced by the peptide substrate at critical mass during the handover of substrate from Hsp40 to Hsp70 (Hennessy *et al.*, 2005). It is therefore possible that the differential influence of co-chaperones on KPf-V436F and PfHsp70-V451F accounted for the disparity in the behaviour of these proteins *in vivo*. Another aspect that could introduce variation of this nature is the close connection between events in the peptide binding domain and their allosteric function (Buchberger *et al.*, 1995; Ha *et al.*, 1997; Montgomery *et al.*, 1999; Pellecchia *et al.*, 2000). Pellecchia *et al.* (2000) observed that chemical shifts originating in the substrate binding cavity are translated to the N-terminus through loops $L_{2,3}$ (Figure 4.1) and vice-versa. In this case a signal coming from a common source such as ATP binding to PfHsp70 or KPf ATPase would naturally manifest a unique signal on the ATPase domain of each chaperone, leading to differential thermodynamics on the hydrophobic pocket.

It has been observed that the contribution of the hydrophobic pocket residue during peptide binding can vary depending on the particular Hsp70. Pfund *et al.* (2001) introduced mutations (valine to phenylalanine switch) in the hydrophobic pockets of yeast Hsp70s, Ssb and its chimeric derivative (composed of the Ssb ATPase domain fused to the peptide binding domain of Ssa, a homologue of Ssb; James *et al.*, 1997). It is interesting to note that

although both proteins had impaired *in vivo* function based on ability to confer cold tolerance and drug resistance to yeast, cells expressing the full length protein Ssb (with the hydrophobic pocket mutation) displayed greater stress sensitivity (Pfund *et al.*, 2001). This could be explained in the context of the fact that Ssa and Ssb have distinct chaperone roles; hence they require unique co-chaperone regulatory systems (James *et al.*, 1997; Pfund *et al.*, 2001). A study on yeast Hsp70, Ssq1 in which a V472F hydrophobic pocket substitution was made led only to a mild *in vivo* phenotypic effect (Knieszner *et al.*, 2005). The data on yeast Hsp70s (Pfund *et al.*, 2001; Knieszner *et al.*, 2005) together with results obtained in this study, suggest that the contribution of the hydrophobic pocket residues in eukaryotic Hsp70s is variable and may differ from its role in prokaryotes. Based on this premise, the PfHsp70 peptide binding domain in KPf-V436F chimera behaves as a prokaryotic Hsp70 segment since it is carrying the *E. coli* DnaK ATPase domain which places the chimeric Hsp70 under the command of the *E. coli* co-chaperone regulatory system. The importance of the ATPase domain in determining the role of Hsp70s is most mirrored in primates where the ATPase domain defines the distinct functions of the constitutive and inducible forms of their Hsp70s (Tutar *et al.*, 2006).

This study establishes that PfHsp70 has a functional substrate binding cavity defined by the arch and hydrophobic pocket. However, mutation of arch residues only reflected minor *in vivo* consequences in PfHsp70 which were masked on the KPf chimera background. Inversely, the contribution of the hydrophobic pocket residue was less important in PfHsp70 than in KPf. Furthermore, data from this study has hinted at the importance of the arch and hydrophobic pocket residues in PfHsp70. Most studies on the role of the substrate binding cavity in Hsp70 proteins (Mayer *et al.*, 2000b; Rüdiger *et al.*, 2000; Knieszner *et al.*, 2005) followed up *in vivo* studies with *in vitro* analysis in order to fully explore the roles of these motifs. Therefore, work covered in Chapter 5 involved the *in vitro* analysis of the chaperone function of PfHsp70 and its substrate binding cavity mutants whose *in vivo* chaperone function was examined in this study.

Chapter Five

In vitro biochemical characterisation of the chaperone properties of PfHsp70

5.1 Introduction

Of particular importance in the study of an Hsp70 chaperone is the investigation of the regulation of its activity by co-chaperone partners and its interaction with peptide substrates. To date, very little work has been done on the *in vitro* analysis of the chaperone properties of PfHsp70 (Matambo *et al.*, 2004; Ramya *et al.* 2006). One aspect of interest is to identify the co-chaperones that regulate the chaperone function of PfHsp70. Not only is this important in order to understand how the function of PfHsp70 is regulated, but the identification of PfHsp70's co-chaperones could reveal the protein network in which it takes part. For example, the fact that PfHsp90 is essential for the parasite's survival (Banumathy *et al.*, 2003), places PfHsp70 into the spotlight since PfHsp70 possesses the EEVD motif; essential for the interaction of Hsp70 with Hsp90 (Freeman *et al.*, 1995; Demand *et al.*, 1998).. The EEVD motif of Hsp70 binds to co-chaperones including Hop, whose role is to facilitate the partnership between Hsp70 and Hsp90 (Freeman *et al.*, 1995; Demand *et al.*, 1998). In their study, Ramya *et al.* (2006) suggested that an antimalarial, 15-deoxyspergulain (DSG), was able to interact with PfHsp70 through its terminal EEVD motif. This indicates that the EEVD motif of PfHsp70 might be useful in its interaction with co-chaperones that act through the C-terminus (Freeman *et al.*, 1995; Demand *et al.*, 1998). Heat shock interacting protein (Hip) is a co-chaperone of Hsp70 which competes with Bag-1 for a contact site in the ATPase domain of Hsp70, and acts in direct opposition to Bag-1 by stabilising the Hsp70-Bag-1-peptide complex (Höhfeld *et al.*, 1995; Höhfeld and Jentsh, 1997). Hip's ability to regulate Hsp70 function *in vivo* has been confirmed (Nollen *et al.*, 2001). Ramya *et al.* (2006) observed that *P. falciparum* Hip (PFE1370w) was able to modulate the chaperone function of PfHsp70 and PfHsp70-3 *in vitro*. There is therefore growing interest in the co-chaperone modulation of PfHsp70 activity.

The ATPase activity of Hsp70 is regulated by Hsp40 co-chaperones (McCarty *et al.*, 1995). *P. falciparum* contains 43 Hsp40-like proteins (Sargeant *et al.*, 2006) and only PfJ1 (type I), PfJ4 (type II); RESA, PfJ2 and PfJ3 (type III) have been partially characterised (Watanabe,

1997). A study based on mRNA expression suggested that PfJ3 is highly heat inducible, whilst PfJ1 and PfJ4 are only moderately heat inducible. Heat stress led to reduced PfJ2 mRNA steady state level (Watanabe, 1997). There is no work that has been carried out to identify the *P. falciparum* Hsp40 co-chaperone of PfHsp70. PfJ4 has been proposed as one of the potential Hsp40 co-chaperones of PfHsp70 (section 2.5). For this reason, the potential modulation of the ATPase activity of PfHsp70 by PfJ4 was analysed in this study. PfJ4 is a type II hsp40 (Watanabe, 1997) of approximately 31.6 kDa.

PfHsp70 has been purified previously based on urea denaturation methods (Matambo *et al.*, 2004; Ramya *et al.*, 2006). This method yields protein whose functional capacity could be compromised because the recovery to a complete native state is not always guaranteed (Kathir *et al.*, 2005). However, Matambo *et al.* (2004) reported a relatively high basal ATPase activity (14.6 nmol/min/mg) for PfHsp70 despite using urea denaturing method for the purification of the protein.

The application of ionic detergents such as polyethylenimine (PEI) and N-laurylsarcosine that facilitate the solubilisation of insoluble proteins is documented (Frankel *et al.*, 1991; Trabbic-Carlson *et al.*, 2004). Thus, these detergents can be used to facilitate the purification of proteins ensuring that they are maintained in native state during the purification. N-laurylsarcosine disrupts hydrophobic interactions that occur during cell lysis involving the target protein (the exogenously expressed protein) and lipolysaccharide and protein fractions of the cell (Osborn and Wu, 1980; Frankel *et al.*, 1991). PEI removes nucleic acids (Trabbic-Carlson *et al.*, 2004), that would normally entangle and complex with the target protein during protein purification.

It is recognised that the role of Hsp70 proteins is not only limited to protein refolding but they also act as 'holdases' that prevent protein aggregation (Skowyra *et al.*, 1990; Slepenkov and Witt, 2002). The suppression of aggregation of model proteins in the presence of molecular chaperones is an assay that has been used by other workers for determining the role of molecular chaperones (Goloubinoff *et al.*, 1999; Manna *et al.*, 2001; Basha *et al.*, 2004; Ramya *et al.*, 2006). Based on one such assay, it was shown that PfHsp70 is able to inhibit protein aggregation *in vitro* (Ramya *et al.*, 2006). However, Ramya *et al.* (2006) carried out most of their work using reduced carboxymethylated lactalbumin (RCMLA) as the model substrate. Although RCMLA interacts with Hsp70 (Bimston *et al.*, 1998), it is not

a good model substrate for use in aggregation prevention assays since its 'folded' state mimics a partially unfolded intermediate, with extensive hydrophobic regions exposed. Instead, malate dehydrogenase (MDH) has been widely used for this purpose (Goloubinoff *et al.*, 1999; Lee and Vierling, 2000; Basha *et al.*, 2004). Therefore, in this study, MDH was used to investigate the chaperone activity of PfHsp70. In addition, the arch and hydrophobic pocket mutants of PfHsp70 (chapter 4) were purified and their ability to inhibit the heat-induced aggregation of MDH was investigated.

The broad objectives of this study were to:

1) Purify PfHsp70 using both urea denaturing and non-denaturing methods.
2) Compare the ATPase activities of PfHsp70 purified by denaturing and non-denaturing means.
3) Express PfJ4 in *E. coli dnaK103* strain, followed by its native purification from the same strain.
4) Investigate whether PfJ4 stimulates the ATPase activity of PfHsp70.
5) Analyse PfHsp70's ability to inhibit heat-induced aggregation of MDH.
6) Examine the effects of arch and hydrophobic pocket substitutions on the ability of PfHsp70 to suppress heat-induced MDH aggregation.
7) To analyse the effect of ATP on PfHsp70 chaperone function.

5.2 Experimental procedures

5.2.1 Materials

A plasmid, pQE30/PfJ4-D80F/N182S, encoding the protein, PfJ4-D80F/N182S, with an N-terminal His-tag was donated by Dr. William Nicol, a former member of our laboratory. This plasmid had two restriction sites (*Bst* BI and *Nru* I) engineered onto it to facilitate the swapping of the J domain of this protein with the same domain from other Hsp40s. This plasmid was codon optimised and codon harmonised to facilitate the expression of PfJ4 in *E. coli* (Nicoll *et al.*, 2006). Other specialised chemical reagents used in this study and their suppliers are listed (Table D.1; Appendix D).

5.2.2 Reversal of PfJ4-D80F/N182S to PfJ4 (wild type) by site-directed mutagenesis

Since the pQE30/PfJ4-D80F/N182S plasmid encoded for PfJ4 with a double substitution on it (D80F/N182S), it was important to reverse the amino acid substitutions to derive a wild type PfJ4. The QuikChange site-directed mutagenesis protocol (Stratagene, U.S.A; Appendix A.6) was used to convert PfJ4-D80F/N182S to PfJ4, by reversing the two mutations. Thus the two restrictions sites (*Bst* BI and *Nru* I) initially present on the pQE30/PfJ4-D80F/N182S plasmid were eliminated upon reversion to pQE30/PfJ4. In order to confirm the changes made on the plasmid encoding PfJ4, diagnostic analysis was carried out using DNA restriction by *Bst* BI and *Nru* I. DNA sequencing (Appendix A.11) was also used to confirm the changes.

5.2.3 Analysis of the expression and solubility of PfJ4 in *E. coli* XL1 Blue and *dnaK103* strains

The plasmid construct encoding pQE30/PfJ4 was used to transform *E. coli* XL1 Blue and *dnaK103* cells, followed by protein expression studies, carried out using a previously described method (section 3.2.5). In order to assess the solubility of PfJ4 in *E. coli* XL1 Blue, a 1 ml sample was taken 5 hours after induction with IPTG. Sampling was done from the overnight culture of *E. coli dnaK103* since this was the stage at which PfJ4 was maximally produced in this strain. The cells were harvested by centrifugation at 5 000g for 20 minutes and resuspended in 1 ml lysis buffer (0.01 mM tris, pH 7.5, 300 mM NaCl, 10 mM imidazole) containing 1 mM phenylmethylsufonyl fluoride (PMSF) and 1 mg/ml of lysozyme. The cells were frozen at -80°C, overnight. The cells were thawed rapidly and then mildly sonicated. The cell lysate was centrifuged at 16 0000 g at 4°C for 20 minutes. The supernatant was collected (soluble fraction) and the pellet was resuspended in 1 ml of phosphate buffered saline (pH 7.5), and this constituted the insoluble fraction. The samples were analysed by SDS-PAGE.

5.2.4 Purification of PfJ4 from *E. coli dnaK103* cells

A colony of *E. coli dnaK103* cells transformed with the pQE30/PfJ4 plasmid was asceptically transferred into 25 ml of 2xYT broth containing 100 µg/ml ampicillin and 10 µg/ml tetracycline and incubated at 30°C overnight. The culture grown overnight was used to inoculate 225 ml of 2xYT broth containing 100 µg/ml ampicillin and 10 µg/ml tetracycline and incubated at 30°C overnight. The cells were harvested by centrifugation at 5 000 g for 20 minutes at 4°C. The pellets were resuspended using 10 ml of lysis buffer containing 1 mM

PMSF and 1 mg/ml lysozyme. The suspended pellet was stored overnight at -80°C. The cells were thawed rapidly and then mildly sonicated. Cell debri was removed by centrifugation at 12 0000 g for 30 minutes at 4°C. The lysate was left to bind to sephadex beads charged with nickel for 4 hours at 4°C. The beads were then washed twice using wash buffers (0.01 mM tris, pH 7.5, 300 mM NaCl, 50 mM imidazole), followed by elution using elution buffer (0.01 mM tris, pH 7.5, 300 mM NaCl, 1 M imidazole). The eluted protein was placed in pre-soaked Snakeskin™ pleated dialysis tubing, M_r 10 000 (Pierce, U.S.A). The protein was dialysed against buffer (0.01 mM tris, pH 7.5, 300 mM NaCl, 50 mM imidazole, 10 % glycerol).

5.2.5 Urea denatured purification of PfHsp70 using nickel affinity chromatography

PfHsp70 was expressed in *E. coli* XL1 Blue and purified using a previously employed protocol involving the use of a urea denaturation step, since the protein has been reported to be insoluble in *E. coli* XL1 Blue cells (Matambo *et al.*, 2004). Cells induced for protein expression were harvested by centrifugation at 5 000 g for 20 minutes at 4°C. The cells were resuspended in denaturing lysis buffer (8 M urea, 300 mM NaCl, 10 mM imidazole, 0.01 mM tris, PH 7.5) containing PMSF (1 mM) and lysozyme (1 mg/ml). The cells were frozen overnight at -80°C. The cells were then thawed and sonicated mildly. To 10 ml of respuspended cells, 1 ml of 50 % (v/v) Ni^{2+}-chelating sepharose beads was added followed by gentle agitation at 4°C for 4 hours. The cell lysate was then centrifuged at 5 000 g for 5 minutes in order to get rid of the unbound protein fraction. This was followed by two wash steps using wash buffer 1 (0.01 mM tris, pH 7.5, 300 mM NaCl, 100 mM imidazole) and wash buffer 2 (0.01 mM tris, pH 7.5, 300 mM NaCl, 150 mM imidazole). The bound protein was then recovered using elution buffer (0.01 mM tris, pH 7.5, 300 mM NaCl, 1 M imidazole). The eluted protein was placed in pre-soaked Snakeskin™ pleated dialysis tubing, M_r 10 000 (Pierce, U.S.A). The protein was dialysed against the dialysis buffer (0.01 mM tris, pH 7.5, 300 mM NaCl, 50 mM imidazole, 10 % glycerol).

5.2.6 Native purification of PfHsp70 and its substrate binding cavity mutants using detergents, PEI and N-lauralysarcosine

The pellet of cells that were harvested for protein purification was resuspended in lysis buffer (0.01 mM tris, pH 7.5, 300 mM NaCl, 50 mM imidazole) containing 1 mM PMSF and 0.1mg/ml lysosyme. Cell lysis was allowed to proceed for 20 minutes, after which the cells were frozen in two batches at -80°C, overnight. The cells were rapidly thawed, and PEI was

added to one batch to a final concentration of 0.1 % (v/v), whilst N-laurylsarcosine was added to the other batch to final concentration of 7.5 % (w/v). The cells were mildly sonicated. Cell debri was removed by centrifugation at 12 0000 g at 4°C for 30 minutes. The PfHsp70 in the cell lysate was left to bind to Ni^{2+}-charged sephadex beads at 4°C for 4 hours. The beads were then washed using wash buffer (0.01 mM tris, pH 7.5, 300 mM NaCl, 50 mM imidazole), followed by elution using elution buffer (0.01 mM tris, pH 7.5, 300 mM NaCl, 1 M imidazole). The eluted protein was placed in pre-soaked Snakeskin™ pleated dialysis tubing, M_r 10 000 (Pierce, U.S.A). The protein was dialysed overnight against dialysis buffer (0.01 mM tris, pH 7.5, 300 mM NaCl, 50 mM imidazole, 10 % glycerol). The subsequent purification of PfHsp70 arch and hydrophobic pocket mutants was carried out using this protocol.

5.2.7 Determination of PfHsp70 ATPase activity

5.2.7.1 Analysis of the basal ATPase activity of PfHsp70 purified by native and urea denaturing methods

The basal ATPase activities of PfHsp70 purified natively and that which was purified by urea denaturation were analysed based on a slightly modified form of the protocol by Matambo *et al.* (2004). A 1200 µl assay mix that had the following constituents in their final concentrations was made: 10 mM HEPES, pH 7.5; 100 mM KCl; 2 mM $MgCl_2$; 0.5 mM dietheothreitol (DTT); and 0.4 µM PfHsp70. The reaction mix was incubated at 37°C for 5 minutes. The reaction was then started by adding ATP to a final concentration of 1 mM. A control experiment monitoring the spontaneous hydrolysis of ATP was set up by having a reaction mix with all the other constituents except PfHsp70 protein. At least three experiments were carried out, using a fresh batch of purified protein each time. Each assay was conducted in duplicate each time the experiment was carried out. Samples (50 µl aliquots) were collected immediately after starting the reaction (0 hour reading) and thereafter at 1 hour intervals for 4 hours. The reaction was stopped by using 50 µl of stop reagent, made up of 10 % (w/v) SDS and 120 mM EDTA. Equal volumes (50 µl each) of 1.25 % (w/v) ammonium molybdate and 9 % (w/v) ascorbic acid were added for the development of a coloured complex whose absorbance was monitored spectrophotometrically at 850 nm. A phosphate standard curve was constructed (Figure C.1; Appendix C) in order to evaluate the amount of phosphate released. The specific ATPase activity was calculated and reported as nmol Pi/min/mg of PfHsp70 protein.

5.2.7.2 Effect of PfJ4 on the ATPase activity of PfHsp70

In order to determine the effect of PfJ4 on the ATPase activity of PfHsp70, purified PfJ4 was added to the reaction mix as described in section 5.2.7.1 to a final concentration of 0.2 μM. The release of Pi was then monitored in the presence of both PfJ4 and PfHsp70 the same way as has been described in section 5.2.7.1.

5.2.8 MDH aggregation suppression assays

This reaction was conducted by monitoring the ability to suppress MDH aggregation by PfHsp70 and its derivatives with substitutions in the substrate binding cavity. All the different proteins that were going to be tested for their ability to prevent MDH aggregation had their stability to heat assessed. This was done by placing 1 ml of assay buffer (20 mM tris, pH 7.4; 100 mM NaCl; Ramya *et al.*, 2006) into a cuvette. The cuvette was then held at 48°C in a UV/Vis spectrophotometer with a temperature control system. The assay buffer was left to equilibrate for 10 minutes before the addition of protein. After the equilibration of assay buffer, the protein whose heat stability was under investigation was added to the assay buffer at a final concentration of 1.3 μM. Turbidimetric changes were then monitored at 360 nm for 30 minutes.

After the heat stability assay was conducted, the proteins were assessed for their ability to prevent MDH aggregation. The assay was conducted as described above. The only difference was that the different proteins were added to the assay buffer together with pig heart MDH (Roche, Germany) at a final concentration of 0.65 μM. The thermally-induced aggregation of MDH was then followed by reading turbidity changes on the UV/Vis spectrophotometer at 360 nm for 30 minutes. For control experiment, bovine serum albumin (BSA) was used in place of PfHsp70 protein. A second control experiment assessed the aggregation of MDH alone (MDH suspended in assay buffer, without any other protein). The assay was repeated at least three times and the experiment was done in duplicate each time. Freshly purified batches of protein were used for each experiment.

5.3 Results

5.3.1 Reversing PfJ4- D80F/N182S to PfJ4 by site-directed mutagenesis

The plasmid construct pQE30/PfJ4-D80F/N182S expressing the protein, PfJ4- D80F/N182S had its integrity confirmed by endonuclease digestion (Figure 5.1). Changes were then effected by site-directed mutagenesis on this plasmid to yield pQE30/PfJ4 plasmid which encoded PfJ4 wild type protein (Figure 5.1). The changes resulted in loss of the two restriction sites; *Bst* BI and *Nru* I. These changes were confirmed by diagnostic endonuclease digestions (Figure 5.1) and through DNA sequencing. The pQE30/PfJ4-D80F/N182S plasmid had sites for *Bst* BI and *Nru* I as shown in lanes 1-4 (Figure 5.1). After reversing the substitutions to get pQE30/PfJ4, these restriction sites were eliminated (Figure 5.1; lanes 6-8). *Pvu* I was used as the diagnostic endonuclease for both plasmids.

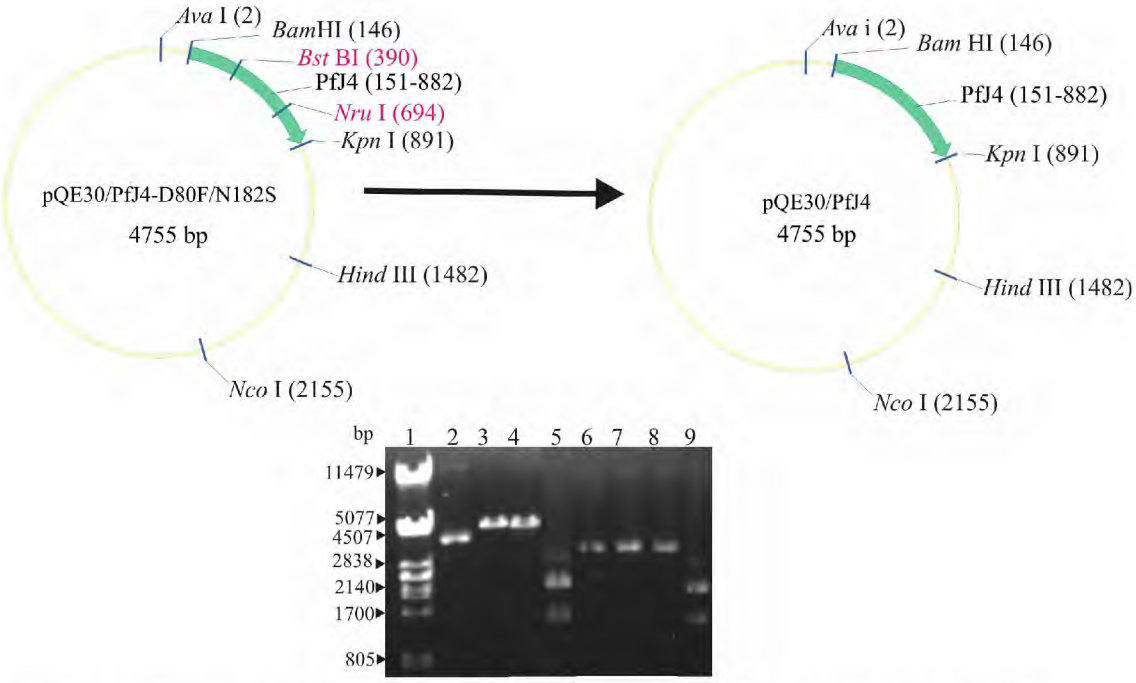

Figure 5.1 Diagnostic restriction analysis of pQE30/PfJ4-D80F/N182S and pQE30/PfJ4 plasmids
Restriction maps (top) and ethidium bromide stained agarose gel for the diagnostic restriction analysis of the plasmids, pQE30/PfJ4-D80F/N182S and pQE30/PfJ4. Lane 1, *Pst* I digested lambda DNA molecular weight standards; lane 2, pQE30/PfJ4-D80F/N182S undigested; lane 3, pQE30/PfJ4-D80F/N182S digested with *Bst* BI; lane 4, pQE30/PfJ4-D80F/N182S digested with *Nru* I; lane 5, pQE30/PfJ4-D80F/N182S digested with *Bam* HI and *Nco* I. Lanes 6-8, represent pQE30/PfJ4 digested under the same conditions presented for pQE30/PfJ4-D80F/N182S in lanes 2-5, respectively. The restriction sites for *Bst* BI and *Nru* I (red) were lost when pQE30/PfJ4-D80F/N182S was changed to pQE30/PfJ4, as shown by arrow.

5.3.2 PfJ4 expression and solubility studies in *E. coli* XL1 Blue strain

The production of PfJ4 in *E. coli* XL1 Blue was confirmed by SDS-PAGE analysis (Figure 5.2). Before induction by IPTG (lane 0; Figure 5.2), no protein band is observed at the expected mobility range (see arrow) of PfJ4 (Figure 5.2). However, upon adding IPTG to the culture, the cells were able to express PfJ4 after 1 hour induction. The production of PfJ4 in *E. coli* XL1 Blue cells was optimal after 4-5 hours of induction (Figure 5.2; lanes 4-5). The solubility study led to the recovery of PfJ4 in the pellet fraction, indicating that the protein was in the insoluble fraction in *E. coli* XL1 cells.

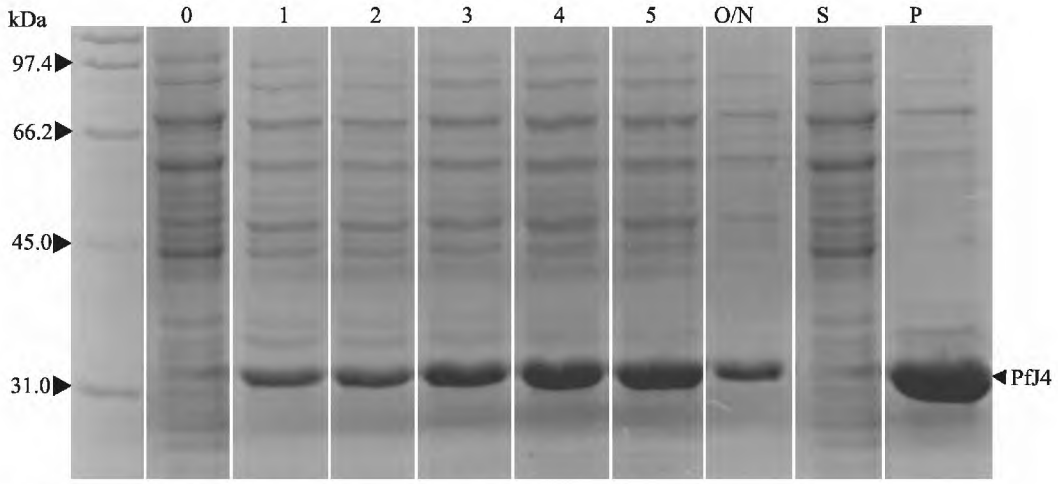

Figure 5.2 Analysis of PfJ4 expression and solubility in *E. coli* XL1 Blue cells
Samples of *E. coli* XL1 Blue cells expressing PfJ4 were taken at different time points during the protein production study and analysed by SDS-PAGE. Lane 0, represents total extract for cells transformed with pQE30/PfJ4 plasmid before IPTG induction; lane 2, 3, 4, and 5, represent total extract for cells transformed with pQE30/PfJ4 plasmid and induced for 2, 3, 4, and 5 hours, respectively; lane O/N, represents total extract for cells transformed with pQE30/PfJ4, after induction overnight; lanes S and P, represent the soluble and pellet fraction for the sample taken 5 hours after induction with IPTG. The arrow labelled 'PfJ4' represents the mobility expected of a $(His)_6$-PfJ4 species. Molecular weight markers (in kDa) are shown in the extreme left hand side lane.

Despite the successful expression of PfJ4 in *E. coli* XL1 Blue cells, the purification of this protein was complicated by the fact that the protein was recovered complexed to the endogenous DnaK protein based on anti-*E coli* DnaK antibody based Western analysis (data not shown). This necessitated the need to employ *E. coli dnaK103* as the heterologous expression system for PfJ4 because this strain has a truncated, non-functional DnaK (Spence *et al.*, 1990).

5.3.3 PfJ4 expression and solubility studies in *E. coli dnaK103* strain

The production of PfJ4 in *E. coli dnaK103* cells was reduced (Figure 5.3) compared to *E. coli XL1* Blue cells (Figure 5.2). However, the lysate of cells that were taken immediately before induction (lane 0) and several hours after induction (lanes 2, 3, 4, 5 and O/N; Figure 5.3) display a band that migrates to the same level as is expected of a his-tagged PfJ4 species (see arrow; Figure 5.3) and this band is not observed in lane 0 (representing cells transformed with the plasmid vector). In addition, the production of PfJ4 was confirmed by Western analysis (Figure 5.3; lower panels). Although the *E. coli dnaK103* cells produced lower amounts of the protein compared to *E. coli* XL1 cells, some of the protein was recovered in a soluble form (Figure 5.3; lanes 'S'). This facilitated the purification of PfJ4 by native means. However, a higher amount of the protein was observed in the pellet fraction (Figure 5.3; lanes 'P').

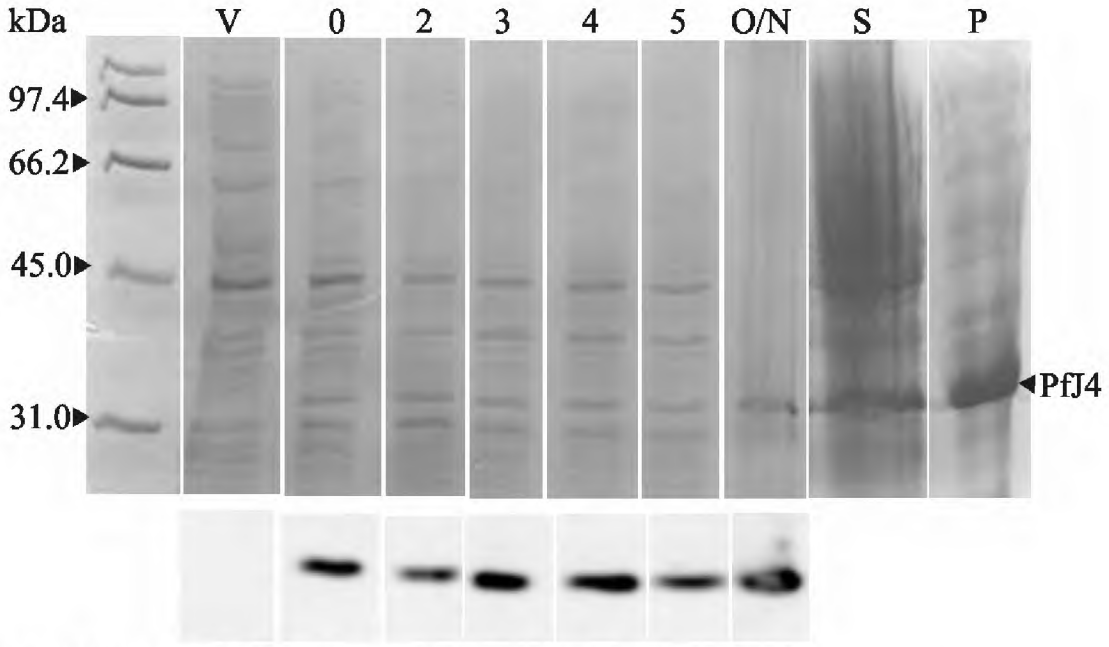

Figure 5.3 Analysis of the expression and solubility of PfJ4 in *E. coli dnaK103*
SDS-PAGE (top panels) and Western (lower panels) analysis for the expression of PfJ4 in *E. coli dnaK103* cells. Samples of *E. coli dnaK103* cells transformed with pQE30/PfJ4 plasmid taken at different time points and analysed by SDS-PAGE. Lane V, represents total extract for cells transformed with pQE30 vector plasmid, lane 0, represents total extract for cells transformed with pQE30/PfJ4 plasmid before IPTG induction; lane 2, 3, 4, and 5, represent total extract for cells transformed with pQE30/PfJ4 plasmid and induced for 2, 3, 4, and 5 hours, respectively; lane O/N, represents total extract for cells transformed with pQE30/PfJ4, after induction overnight; lanes S and P, represent the soluble and pellet fraction for the sample taken after induction overnight. The arrow labelled 'PfJ4' represents the mobility expected of a $(His)_6$-PfJ4 species. Molecular weight markers (in kDa) are shown in the extreme left hand side lane.

5.3.4 Native purification of PfJ4 expressed in *E. coli dnaK103* cells

By expressing PfJ4 in *E. coli dnaK103* cells, it was possible to recover soluble protein (Figure 5.3), making it possible to purify the protein in native form (Figure 5.4). There was minimal loss of the protein through the wash step (Figure 5.4; lane W), resulting in most of the protein being eluted (lanes E1 and E2). The eluted protein was dialysed to remove the imidazole that was used during the elution steps (Figure 5.4; lane BE).

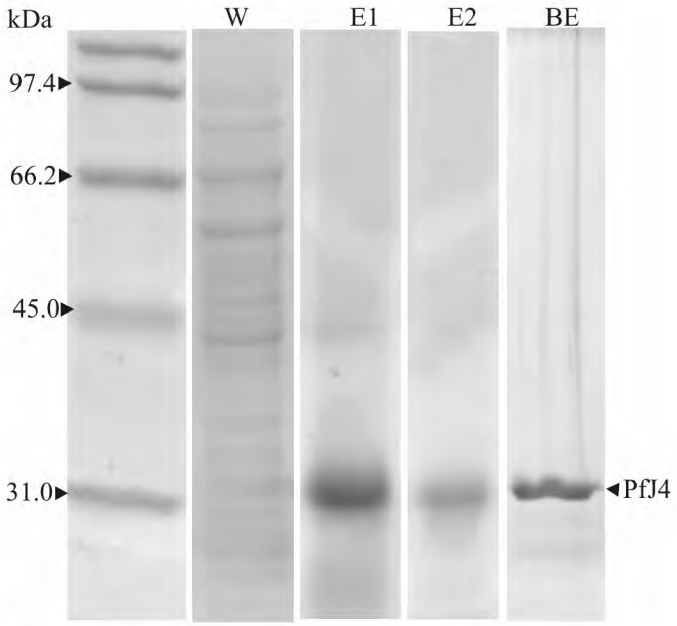

Figure 5.4 Native purification of PfJ4 by Nickel affinity chromatography
SDS-PAGE analysis confirming the successful native purification of PfJ4 overproduced in *E. coli dnaK103* cells. Lane W, represents wash step; lanes E1 and E2, represent first and second elution steps, respectively (1M imidazole in tris-HCl buffer was used for elution); and lane BE, represents total eluted protein recovered after buffer exchange. The arrow labelled 'PfJ4' represents the mobility expected of a $(His)_6$-PfJ4 species. Molecular weight markers (in kDa) are shown in the extreme left hand side lane.

5.3.5 Urea denaturation purification of PfHsp70

The heterologous overproduction of PfHsp70 in *E. coli* and its purification has been previously optimised (Matambo *et al.*, 2004; Ramya *et al.*, 2006). Based on a previously employed protocol used by (Matambo *et al.*, 2004), it was possible to purify PfHsp70 (Figure 5.5). Since the protein has been recovered in the pellet fraction of the *E. coli* cells, this protocol involves a urea denaturing step of the protein followed by native washes and elutions (Matambo *et al.*, 2004). Although some of the protein was lost during the wash steps (Figure 5.5; lanes W1 and W2), there was a substantial amount of the protein recovered upon elution (Figure 5.5; lanes E1 and E2).

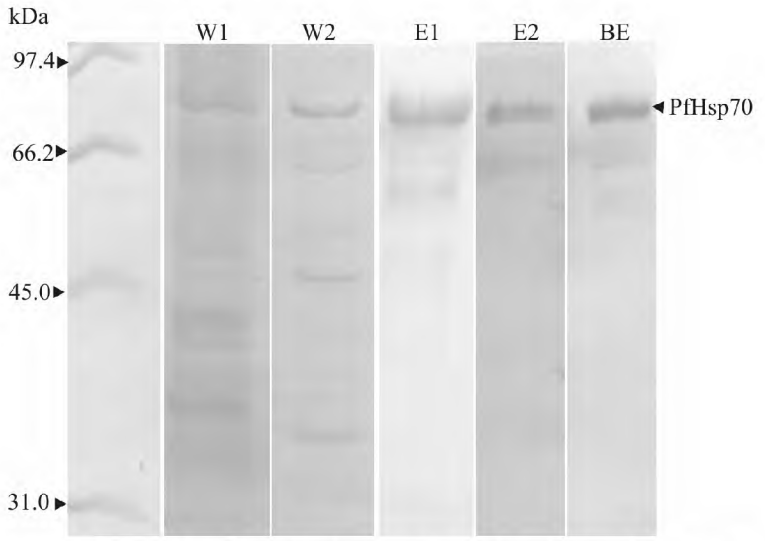

Figure 5.5 Urea denaturation purification of PfHsp70 using Nickel affinity chromatography
SDS-PAGE analysis representing PfHsp70 purification using Nickel affinity chromatography. Lanes W1 and W2, represent washes 1 and 2; lanes E1 and E2, represent first and second elution steps, respectively (1 M imidazole in tris-HCl buffer was used for elution) and 2; and lane BE, represents total eluted protein recovered after buffer exchange. Urea denaturation was applied, followed by native wash and elution steps during the purification. The arrow labelled 'PfHsp70' represents the mobility expected of a $(His)_6$-PfHsp70 species. Molecular weight markers (in kDa) are shown in the extreme left hand side lane.

5.3.6 Native purification of PfHsp70 using PEI and N-laurylsarcosine

Since protocols previously used to purify PfHsp70 involved a urea denaturing step, with native washes and elutions (Matambo *et al.*, 2004; Ramya *et al.*, 2006), it was important to attempt a purification protocol in which the protein is maintained in native state throughout the purification process. Using 0.1 % (v/v) PEI and 7.5 % (w/v) N-laurylsarcosine, it was possible to purify PfHsp70 in native form (Figure 5.6). However, the recovery of the protein was better using PEI compared to N-laurylsarcosine. Most of the protein was lost in the wash step (Figure 5.6; lane W) when 7.5 % (w/v) N-laurylsarcosine was used in the purification. Overall, through the use of 0.1 % PEI, it was possible to recover an average of 2 mg of protein from 1000 ml of culture. Therefore, 0.1 % PEI was used during all subsequent PfHsp70 purifications, and the term 'natively purified PfHsp70' will be used to describe PfHsp70 protein purified by this method.

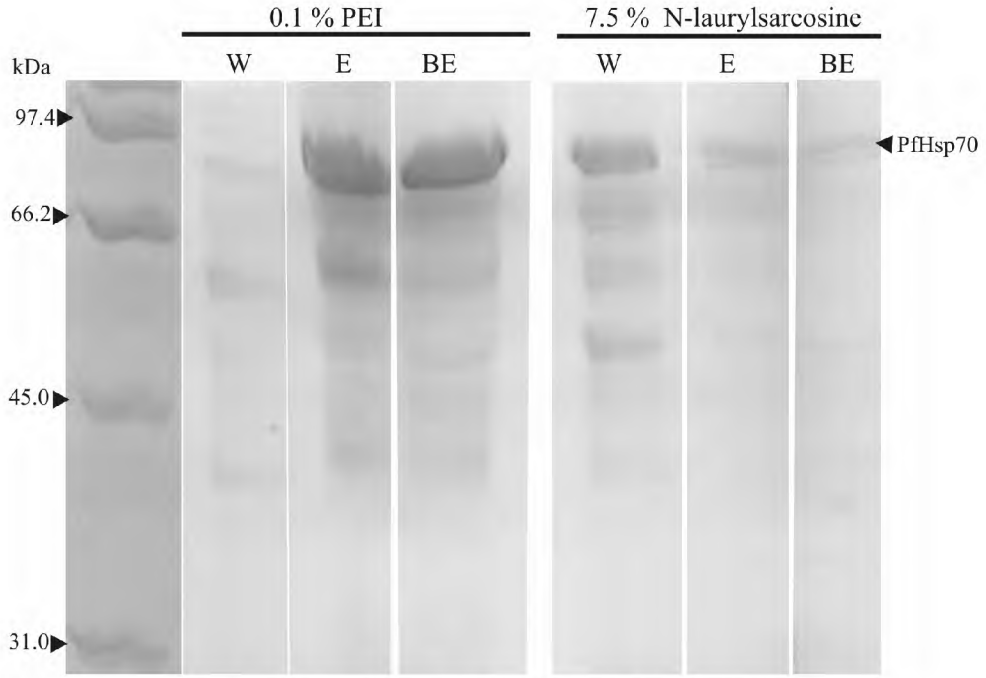

Figure 5.6 Native purification of PfHsp70 using Nickel affinity chromatography
SDS-PAGE analysis for the native purification of PfHsp70. Through the use of 0.1 % (v/v) PEI and 7.5 % (w/v) N-laurylsarcosine, the protein was preserved in native form throughout the purification. Lanes representing samples for each of the two purification methods are as follows: lane W, represents wash sample; lane E, elution sample (1 M imidazole in tris-HCl buffer was used for elution); and lane BE, eluted protein recovered after buffer exchange. The arrow labelled 'PfHsp70' represents the mobility expected of a $(His)_6$-PfHsp70 species. Molecular weight markers (in kDa) are shown in the extreme left hand side lane.

5.3.7 PfJ4 failed to stimulate the ATPase activity of PfHsp70

In this study PfHsp70 purified through urea denaturing step had a basal activity of 10.4 nmol Pi/min/mg (Figure 5.7). A previous report of the basal ATPase activity of PfHsp70 protein that was purified by a similar method was given as 14.6 nmol Pi/min/mg (Matambo *et al.*, 2004). Natively purified PfHsp70 had a specific basal activity of 29.2 nmol Pi/min/mg. Thus, PfHsp70 purified natively through the use of PEI had approximately three times the ATPase activity of the same protein purified through the urea denaturation step (Figure 5.7). PfJ4 did not show detectable influence on the ATPase activity of PfHsp70 purified either natively or through the urea denaturation protocol (Figure 5.7). Instead, in the presence of PfJ4, the ATPase activity of PfHsp70 was marginally suppressed, with ATPase activities of 7.4 nmol Pi/min/mg and 27.3 nmol Pi/min/mg for the protein purified through urea denaturation and the natively purified protein, respectively.

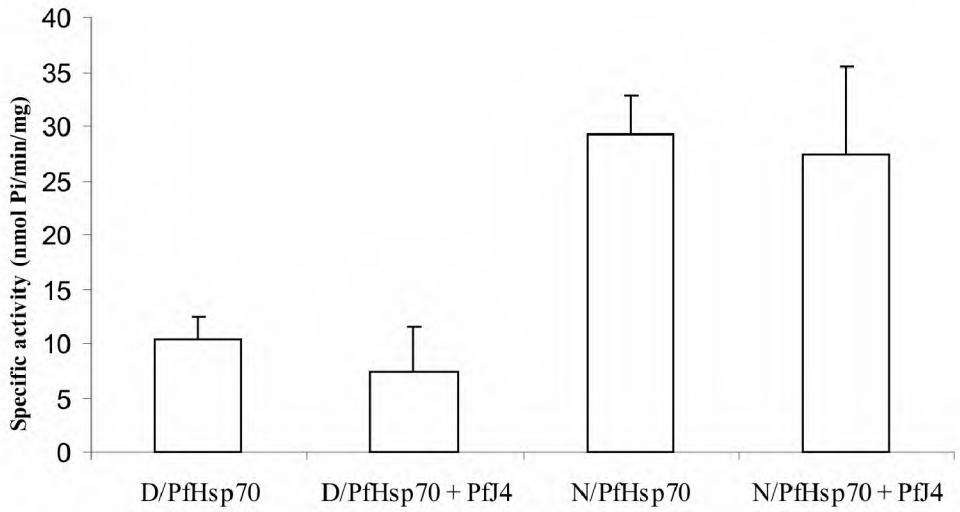

Figure 5.7 Analysis of the ATPase activity of PfHsp70 in the absence and presence of PfJ4
PfHsp70 (0.4 μM) purified through urea denaturation followed by native washes and elution (D/PfHsp70) had its basal ATPase activity analysed in comparison to that of natively purified protein (N/PfHsp70). The ATPase activities of these batches of PfHsp70 were then analysed in the presence of 0.2 μM PfJ4. The graphs represent data obtained from three independent experiments (each experiment was run in duplicate). The mean specific activities obtained are represented by the vertical bar graphs and the respective standard errors of the mean are also indicated.

5.3.8 Native purifications of PfHsp70 arch and hydrophobic pocket mutants

PfHsp70 arch and hydrophobic pocket mutants (PfHsp70-A419Y, PfHsp70-Y444A, PfHsp70-A419Y/Y444A) and PfHsp70-V451F) were produced in *E. coli* XL1 Blue cells and purified using 0.1% PEI (Figure 5.8) in the same way as PfHsp70 (wild type protein) (section 5.3.6). The proteins were thus successfully purified in their native forms.

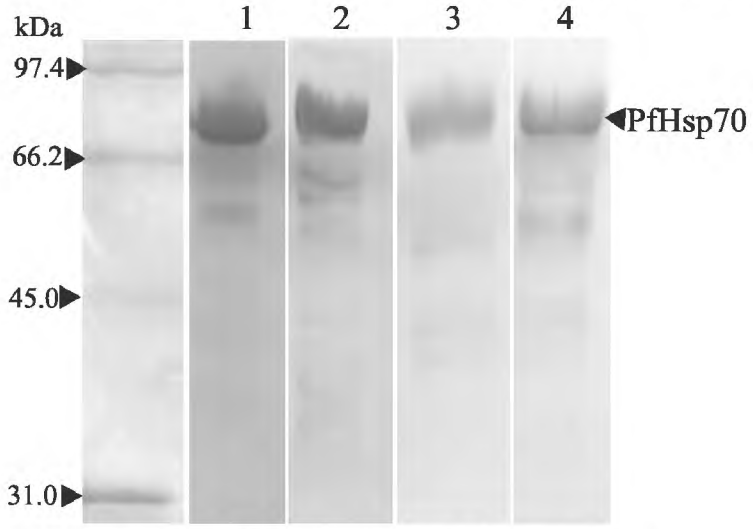

Figure 5.8 Native purification of PfHsp70 arch and hydrophobic pocket variants
SDS-PAGE analysis for the native purification of PfHsp70 arch and hydrophobic pocket mutants through the use of 0.1 % PEI. Lane 1, purified PfHsp70-A419Y; lane 2, PfHsp70-Y444A; lane 3, PfHsp70-A419Y/Y444A; and lane 4, PfHsp70-V451F. The arrow labelled 'PfHsp70' represents the mobility expected of a $(His)_6$-PfHsp70 species. Molecular weight markers (in kDa) are shown in the extreme left hand side lane.

5.3.9 Evaluation of the heat stability of purified of PfHsp70 and its substrate binding cavity variants

PfHsp70 and its variants with amino acid changes in the substrate binding cavity were assessed for their ability to suppress the heat-induced aggregation of MDH. It was important to test the purified forms of these proteins to verify that they were heat stable before assessing their ability to suppress MDH aggregation. Control experiments in which BSA was challenged by heat showed that this protein did not aggregate due to heat stress at 48°C (Figure 5.9). On the other hand, exposing MDH (0.65 μM) to heat, either alone, or in the presence of BSA (1.3 μM), resulted in increased turbidity (up to approximately 0.30 absorbance units) over 30 minutes (Figure 5.9). The increase in turbidity observed when MDH was heated alone (with no other protein suspended in the reaction mix) was attributed to its aggregation. Similarly, the increase in turbidity observed upon exposing MDH to heat in the presence of BSA is attributed MDH aggregation since the control reaction in which BSA suspended in assay buffer was heated at 48°C did not register a notable increase in turbidity.

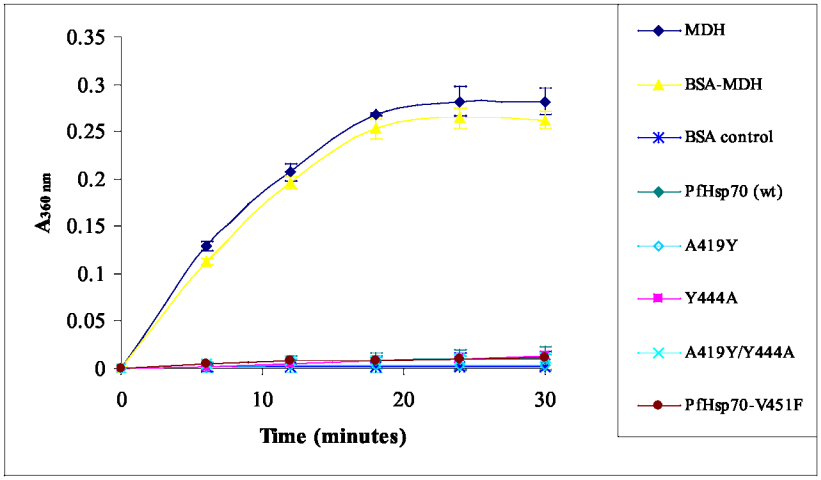

Figure 5.9 Heat stability assay of BSA, purified PfHsp70 and its derivatives
Pig heart MDH (0.65 μM) was suspended in assay buffer either alone (MDH), or in the presence of BSA (1.3 μM) (BSA-MDH). Heat-induced MDH aggregation (increase in turbidity) was followed at 48°C using a spectrophotometer at 360 nm. As control, the aggregation of BSA (BSA control) and PfHsp70 (wild type) as well as its arch and hydrophobic pocket variants were evaluated under the same conditions. In the control experiment, BSA and purified PfHsp70 (or its substrate binding cavity variants) were suspended in assay buffer at a concentration of 1.3 μM in the absence of MDH and their stability to heat was monitored by following the change in absorbance at 48°C for 30 minutes. The graphs represent data obtained from three independent experiments (each experiment was run in duplicate). The standard errors about the mean for each graph are shown.

To test whether PfHsp70 protein or its variants were heat stable, the proteins were suspended in assay buffer and then exposed to heat at 48°C over 30 minutes (Figure 5.9). No significant increase in turbidity was observed when these proteins were exposed to heat at 48°C, suggesting that they were heat stable at this temperature. Therefore, any increase in turbidity that would be observed for test samples containing any of these proteins in the presence of MDH would be due to MDH aggregation.

5.3.10 PfHsp70 suppressed MDH aggregation in dose-dependent fashion

In this study, the ability of PfHsp70's to suppress MDH aggregation was investigated. As expected, MDH aggregation was observed with time when it was exposed to heat, either alone, or in the presence of BSA (Figure 5.10). When PfHsp70 (0.325 μM) was used instead of BSA, there was a reduction in turbidity compared to a scenario in which MDH was heated either alone, or in the presence of BSA. When the concentration of PfHsp70 was increased to 0.65 μM, a further drop in turbidity was observed. This trend (decrease in turbidity) was further enhanced when a higher concentration of PfHsp70 (1.3 μM) was used. Therefore the extent to which MDH aggregation was suppressed depended on the concentration of PfHsp70 in the reaction mix.

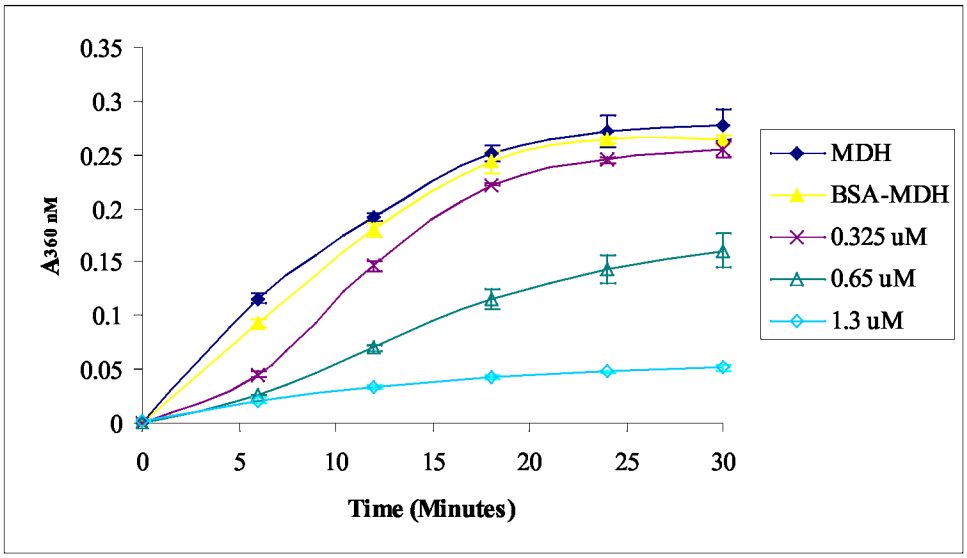

Figure 5.10 Dose-dependent suppression of MDH aggregation by PfHsp70
The aggregation of MDH alone (MDH) or in the presence of BSA (BSA-MDH) was followed at 48°C at 360 nm for 30 minutes. In order to monitor the ability of PfHsp70 to suppress MDH aggregation, different reaction mixes containing 0.65 μM of MDH suspended in assay buffer in the the presence of different concentrations of PfHsp70 (0.325 μM, 0.65 μM and 1.3 μM) were set up. The aggregation of MDH in the presence of varying amounts of PfHsp70 was monitored by measuring the change in turbidity at 48°C at 360 nm for 30 minutes. The graphs represent data obtained from three independent experiments (each experiment was run in duplicate). Variations of standard error about the means are shown on each graph.

5.3.11 PfHsp70 suppressed MDH aggregation in an ATP-dependent mechanism

In this experiment, MDH (0.65 μM) was heat-treated in the presence of BSA (1.3 μM) or alone and the reaction was allowed to proceed over time. After 27 minutes, ATP (1 mM final concentration) was added and turbidity was monitored over the next 3 minutes (Figure 5.11). There was no change in turbidity that was observed when ATP was added. When the same experiment was repeated in the presence of MDH (0.65 μM) and PfHsp70 (1.3 μM), a sharp increase in turbidity was observed immediately when ATP (1 mM) was added (Figure 5.11). The increase in turbidity levelled off at approximately 0.27 A_{360} after 3 minutes. This is nearly the same maximum level of turbidity that was observed for MDH heat-treated either alone, or in the presence of BSA (Figure 5.11). This increase in turbidity was due to MDH aggregation as PfHsp70 released it in response to the addition of ATP.

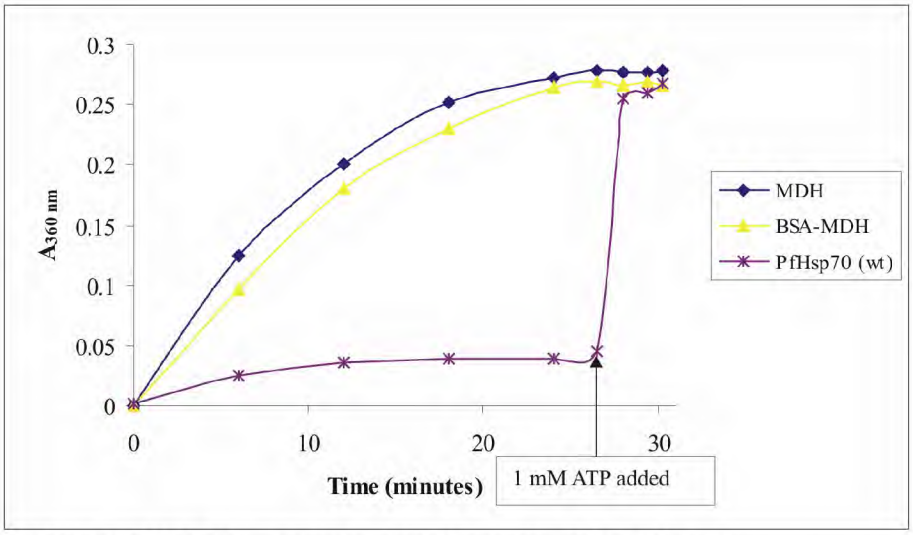

Figure 5.11 Effect of ATP on the suppression of MDH aggregation by PfHsp70
The aggregation of 0.65 μM MDH alone (MDH), or in the presence of 1.3 μM BSA (BSA-MDH) or 1.3 μM PfHsp70 was monitored at 48°C using a spectrophotometer set at 360 nm for 30 minutes. After 27 minutes, ATP was added to a final concentration of 1 mM in all the three reactions. The addition of ATP to the reaction mix containing MDH and PfHsp70, led to a steep rise in turbidity from around 0.04 to approximately 0.27 absorbance units in 3 minutes. No change in absorbance was observed when the same amount of ATP was added to thecontrol reaction mix containing either 0.65 μM MDH supended in assay buffer alone (MDH) or in the presence of 1.3 μM BSA (BSA-MDH).

5.3.12 The effect of arch and hydrophobic pocket mutations on the ability of PfHsp70 to suppress MDH aggregation

It has been shown that amino acid substitutions involving residues of the substrate binding cavity constituting the arch and hydrophobic pocket of PfHsp70 and KPf chimera revealed functional deficiencies of varying degrees based on complementation assays (chapter 4). Using MDH aggregation suppression assays, the effects of these changes on the *in vitro*

chaperone function of PfHsp70 were investigated. Reactions mixtures containing MDH (0.65 μM) and either BSA or PfHsp70 wild type/mutant protein (1.3 μM) were set up and the ability of the different proteins to inhibit MDH aggregation was followed (Figure 5.12). As previously observed, MDH exposed to heat either alone, or in the presence of BSA was not protected from heat-induced aggregation (Figure 5.12). PfHsp70 (wild type) protein was able to inhibit the aggregation of MDH as previously observed (Figure 5.10). Though less effective than the wild type protein, PfHsp70-Y444A was able to suppress MDH aggregation. On the other hand, PfHsp70-A419Y displayed large functional deficiencies in suppressing heat-induced MDH aggregation. PfHsp70-A419Y/Y444A (with arch is inverted compared to the wild type protein), though marginally more effective in suppressing MDH aggregation than PfHsp70-A419Y, failed to protect MDH aggregation compared to wild type protein. The PfHsp70-V451F derivative was less effective in suppressing MDH aggregation compared to wild type protein and the PfHsp70-Y444A derivative, but was more effective than the mutants carrying the A419Y change.

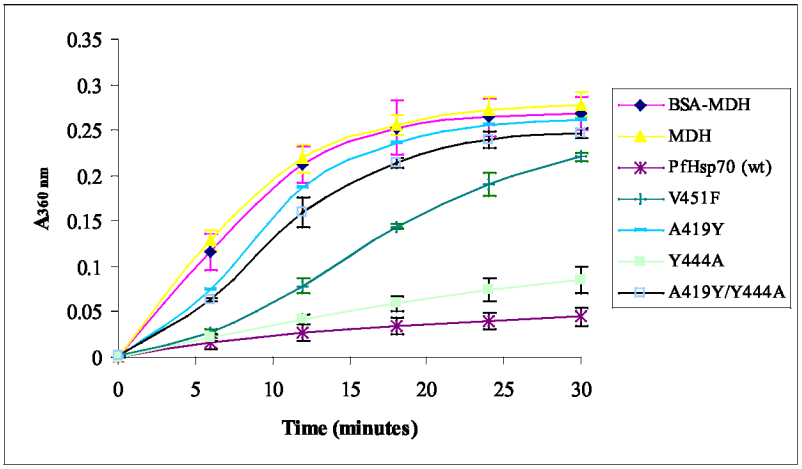

Figure 5.12 Effects of arch and hydrophobic pocket mutations on the ability of PfHsp70 to suppress MDH aggregation

The aggregation of 0.65 μM MDH alone (MDH) or in the presence of 1.3 μM BSA (BSA-MDH) was moniotored spectrophotometrically at 48°C for 30 minutes. The experiment was repeated in the presence of 0.65 μM MDH and 1.3 μM PfHsp70 or its variants (PfHsp70-V451F, PfHsp70-A419Y, PfHsp70-Y444A and PfHsp70-A419Y/Y444A) in order to investigate the ability of the different PfHsp70 variants to suppress MDH aggregation.

The widened gap in the arch of PfHsp70-Y444A would be expected to improve MDH binding by this protein, thus protecting it from heat-induced aggregation. In contrast, the narrower arch in PfHsp70-A419Y (section 4.3.1; Figure 4.2) must have reduced the affinity of this protein for MDH, exposing MDH to heat-induced aggregation. The A419Y/Y444A

possessed an inverted arch compared to the original structure. The fact that PfHsp70-A419Y/Y444 failed to protect MDH from the heat-induced aggregation suggests that the inversion of the arch may have compromised its ability to bind MDH. The PfHsp70-V451F whose hydrophobic pocket was partially blocked by the introduction of the large phenylalanine residue (section 4.3.1; Figure 4.2) showed reduced ability to protect MDH aggregation compared to wild type protein. However, this protein was more effect in inhibiting MDH aggregation compared to PfHsp70-A419Y and PfHsp70-A419Y/Y444A.

5.4 Discussion

Previous studies on the biochemical characterisation of PfHsp70 used urea denaturation purified protein (Matambo *et al.*, 2004; Ramya *et al.*, 2006). It is conceivable that purification of PfHsp70 through urea denaturation might compromise its full recovery to native form, leading to an underestimation of its functional activity. Using natively purified protein, this study attempted to investigate whether PfJ4, a type II Hsp40, functionally interacts with PfHsp70. In addition, this study also analysed the effect of arch and hydrophobic pocket amino acid substitutions on the function of PfHsp70.

PfHsp70 has been reported to occur in the pellet fraction during fractionation studies (Matambo *et al.*, 2004). However, data from the present study suggests that the protein is produced in a soluble form in *E. coli* XL1 Blue cells. The fact that both PEI and N-laurylsarcosine were able to facilitate the recovery of the protein in the soluble and native form (Figure 5.6) indicates that the protein must have been in the soluble form in the *E. coli* cells before the purification started. PEI and N-laurylsarcosine inhibit aggregation involving cell debri (RNA, liposaccharides, protein fractions of the outer membrane) and target protein (Osborn and Wu, 1980; Frankel *et al.*, 1991; Trabbic-Carlson *et al.*, 2004). Therefore the presence of PEI and N-laurylsarcosine during PfHsp70's purification, must have led to the preservation of PfHsp70 in its soluble form. Compared to the use of N-laurylsarcosine, the purification of PfHsp70 in the presence of PEI led to better recovery of the protein in its native form (Figure 5.6; lanes E and BE). Because in the previous method employed in the purification of PfHsp70 (Matambo *et al.*, 2004), there was no attempt to prevent the association of PfHsp70 with cell debri during its purification, the protein must have been entangled in the pellet fraction of the cell.

PfHsp70 purified through urea denaturation was reported to have a basal ATPase activity of 14.6 nmol Pi/min/mg (Matambo *et al.*, 2004). In this study, PfHsp70 purified through the same way was found to have a lower ATPase activity of 10.4 nmol Pi/min/mg (section 5.3.7). PfHsp70, maintained in its native form through all the purification steps in the presence of PEI, had a basal ATPase activity of 29.2 nmol Pi/min/mg (Figure 5.7). Therefore, it is likely that the urea denaturation purification procedure was not accompanied by a full recovery of the PfHsp70 to its complete native form. Using 6.9 M GuHCl as denaturant, Maxwell *et al.* (2003) showed that proteins purified through denaturing purification, followed by dialysis allowed proteins to recover their three-dimensional state, however, this study was only based on small proteins (<18 kDa). There is evidence that the recovery of proteins from the effects of chemical denaturation is not always complete, and the recovery success depends on the length of time the protein would have been exposed to the denaturant (Kathir *et al.*, 2005). Based on data obtained in this study, urea denaturation of PfHsp70 does not allow complete functional recovery of this protein. The observed basal ATPase activity of natively purified PfHsp70 (29.2 nmol Pi/min/mg) is relatively high compared to values obtained for Hsp70s from other species such as human (2.0 nmol Pi/min/mg; Brimston *et al.*, 1998), bovine Hsc70 (2.9 nmol Pi/min/mg; O'Brien *et al.*, 1998) and *E. coli* DnaK (3.5 nmol Pi/min/mg; Liberek *et al.*, 1991). Another parasitic Hsp70, *Trypanosma cruzi* Hsp70 has been reported to have high basal ATPase activity, varying from 60 nmol Pi/min/mg (Edkins *et al.*, 2004) to 550 nmol Pi/min/mg (Olson *et al.*, 1994); suggesting that Hsp70s of parasitic origin may have comparably high basal ATPase activities. This study and previous analysis of the ATPase activity of PfHsp70 (Matambo *et al.*, 2004) relied on the use of N-terminal His-tags to facilitate protein purification. It would be more reliable to use non-tagged Hsp70 protein for the analysis of its activity. However, a previous study showed that hamster BiP, displayed the same ATPase activity in the absence and presence of its N-terminal His-tag (Wei and Hendershot, 1995).

PfJ4 was successfully overproduced in *E. coli* XL1 Blue but, however, it occurred in the insoluble protein of the cell (Figure 5.2), hence it was necessary to use a denaturing method for its purification. Purification of PfJ4 from *E. coli* XL1 Blue cells was further problematic since PfJ4 co-purified with the endogenous DnaK (data not shown). The *E. coli dnaK103* strain which contains a truncated and non-functional DnaK (Spence *et al.*, 1990), was an ideal alternative expression system. PfJ4 expression in *E. coli dnaK103* cells, led to reduced expression of the protein than was observed in *E. coli* XL1 Blue (Figures 5.2 and 5.3).

However, the *E. coli dnaK103* cells were able to produce enough protein to ensure satisfactory purification (Figure 5.4). The added advantage of using *E. coli dnaK103* cells for the expression of PfJ4 was the fact that a fraction of the protein was produced in a soluble form (Figure 5.3; lane 'S'), leading to its successful purification in native state (Figure 5.4). In addition, the expression of PfJ4 in *E. coli dnaK103* and its purification from this strain, circumvented the possible co-purification of endogenous DnaK with the heterologously produced PfJ4 since this strain contains a truncated, non-functional DnaK (Spence *et al.*, 1990).

PfHsp70 possesses a potential Hsp40-binding cleft in its ATPase domain (section 2.3.2.5). However, none of the 43 Hsp40-like proteins of *P. falciparum* (Sargeant *et al.*, 2006), has been established as PfHsp70's co-chaperone partner. Because it is the type I and II Hsp40 proteins only that can interact with Hsp70 as well as bind substrate (Walsh *et al.*, 2004), it is likely that PfHsp70 would have a type I or II Hsp40 partner. PfJ4 is a type II Hsp40 protein, whose mRNA expression is moderately heat inducible, although it is unknown whether this is accompanied by the production of the protein in *P. falciparum* (Watanabe, 1997). It was important to investigate whether PfJ4 can modulate the ATPase activity of PfHsp70. Studies conducted using PfHsp70 purified through both urea denaturation and native purification, showed that PfJ4 does not modulate the ATPase activity of PfHsp70 (Figure 5.7). Instead, in the presence of PfJ4 a slight decrease in ATPase activity of PfHsp70 was observed (Figure 5.7), although the statistical significance was not ascertained. Alternatively, PfJ4 may have been masked by the relatively high basal ATPase activity of natively purified PfHsp70 obtained in this study. It is possible that PfJ4 is not the Hsp40 partner of PfHsp70 since other possible candidates for this role have been discussed previously (section 2.5). Since the basal ATPase activity of PfHsp70 observed in this study was higher than previously observed for *E. coli* DnaK (Liberek *et al.*, 1991) and human Hsc70 (Brimston *et al.*, 1998), it is possible that PfHsp70 may have co-purified with a contaminant which had ATPase activity. The possible presence of such a contaminant in the ATPase activity assay mix could be responsible for the elevated ATPase activity observed which may have masked the effect of PfJ4 on the ATPase activity of PfHsp70.

This study supports the recent observation by Ramya *et al.* (2006) confirming that PfHsp70 has the ability to inhibit protein aggregation (Figure 5.10). Ramya *et al.* (2006) used RCMLA as the model substrate for the main part of their study, whilst MDH was used in this study. It

was hoped that the use of MDH would be more reliable since RCMLA mimics the unfolded state and is bound by Hsp70 even in the absence of heat stress (Ivey *et al.*, 2000). This makes RCMLA unsuitable for aggregation prevention assays since an ideal substrate for this assay should unfold largely in response to the effect of heat during the course of the assay. Although MDH is not a *P. falciparum* protein, it was used as a model substrate of PfHsp70 based on the assumption that Hsp70 proteins are able to recognise Hsp70 binding sites that occur at a frequency of every 36 residues in all proteins (Rüdiger *et al.*, 1997). The observed reduction in the aggregation of MDH when PfHsp70 concentration was increased (Figure 5.10), confirmed that PfHsp70 was responsible for the prevention of MDH aggregation.

MDH aggregation suppression assays were conducted to investigate the role of PfHsp70 arch and hydrophobic pocket residues in its aggregation inhibiting role (section 5.3.12). The arch and hydrophobic pocket variants exhibited varying degrees of functional deficiencies compared to the wild type protein (Figure 5.12). PfHsp70-Y444A inhibited MDH aggregation marginally less effectively than the wild type protein. PfHsp70-Y444A could have failed to suppress MDH aggregation as did the wild type protein because the widened gap in its arch may have promoted the access of substrate (MDH) into the substrate binding cavity as well as promoting faster release of substrate from the substrate binding cavity. The faster substrate off rate for PfHsp70-Y444A may have compromised its ability to prevent MDH aggregation compared to the wild type PfHsp70 protein. PfHsp70-A419Y completely lost its ability to inhibit heat-induced MDH aggregation. PfHsp70-A419Y/Y444A, had its function severely affected, although it was able to suppress MDH aggregation better than PfHsp70-A419Y. PfHsp70-V451F was not able to prevent MDH aggregation compared to wild type protein but was more functionally effective than PfHsp70-A419Y and PfHsp70-A419Y/Y444A.

The A419Y substitution could have partially blocked access of the binding cavity of PfHsp70 by MDH (section 4.3.1; Figure 4.2); leading to its failure to prevent MDH aggregation (Figure 5.12). Generally, these results are congruent with the *in vitro* substrate affinity data obtained for similar substitutions in DnaK (Rüdiger *et al.*, 2000). Studies by Rüdiger *et al.* (2000) showed that DnaK-A429W and DnaK-M404A/A429W variants (similar to PfHsp70-A419Y and PfHsp70-A419Y/Y44A) exhibited widely divergent affinities for the peptide λ CI-F160-Q172 compared to wild type protein. Therefore, the A419Y substitution could have reduced PfHsp70's affinity for MDH as was observed for its equivalence in DnaK (A429W)

(Rüdiger *et al.*, 2000). The reduced functional capability exhibited by the arch inversion mutation in PfHsp70-A419Y/Y444A probably is due to the fact that PfHsp70 may have failed to bind MDH in this orientation. It is known that DnaK binds its substrate in a specific orientation, and reversal of the orientation of its substrates has a negative effect on DnaK's ability to recognise its substrates (Rüdiger *et al.*, 2001). Inversely, the reversal of the position of the arch residues could impair an Hsp70's ability to recognise some of its substrates.
In DnaK, the hydrophobic pocket substitution (V436F) incurred the most drastic loss of affinity for substrates (Rüdiger *et al.*, 2000). PfHsp70-V451F failed to prevent MDH aggregation, albeit, its *in vitro* activity was better than that of PfHsp70-A419Y and PfHsp70-A419Y/Y444A. Data generated from other studies on the role of the hydrophobic pocket of Hsp70s from different species (Mayer *et al.*, 2000b; Rüdiger *et al.*, 2000; Pfund *et al.*, 2001; Knieszner *et al.*, 2005, section 4.4) highlights the fact that the role of the hydrophobic pocket is of variable importance across the different species, with its role in eukaryotes perhaps less important than in prokaryotes.

The role of the arch and hydrophobic pocket residues of Hsp70s varies depending on the binding motifs that the particular Hsp70 recognises. HscA, an *E. coli* Hsp70 homologue, recognises a distinct binding motif different from that of DnaK (Rüdiger *et al.*, 1997; Hoff *et al.*, 2002). However, its arch residues (M401 and F426) do not seem to have a big influence on substrate selectivity since HscA with an F426A substitution displayed a largely similar peptide binding pattern to that of wild type protein (Tapley *et al.*, 2006). On the other hand, the hydrophobic pocket residue (M433) of HscA is important for substrate selection (Tapley *et al.*, 2006). This is in contrast to the role of its equivalent residue (V436) in DnaK, whose role is mainly the regulation of affinity for substrate, and not substrate selection (Mayer *et al.*, 2000b; Rüdiger *et al.*, 2000). In addition, other residues in the substrate binding cleft of Hsp70 proteins, besides the arch and hydrophobic pocket residues, are thought to contribute towards substrate specificity as well (Tapley *et al.*, 2006). This suggests that the poorly conserved residues in the peptide binding cleft of Hsp70s may play an important role in the determination of substrate specificity. Therefore, the fact that the hydrophobic pocket residue of PfHsp70 was able to bind MDH (as evidenced by its marginal ability to suppress MDH aggregation) may be indication that the contribution of this residue is less important in PfHsp70 than in DnaK. The complexicity of peptide binding by Hsp70s is further highlighted by BiP (the ER-based Hsp70), which is able to distinguish between peptides that fold slowly and those that are able to attain their native state faster (Hellman *et al.*, 1999).

The fact that PfHsp70 arch and hydrophobic pocket mutants inhibited MDH aggregation *in vitro* less effectively than the wild type protein confirms that PfHsp70 is able to interact with model substrates through its arch and hydrophobic pocket residues in a typical chaperone fashion and not by a mere passive process. There is no study that has been carried out that shows PfHsp70's direct involvement in peptide binding in *P. falciparum*. Because this study confirmed the ability of PfHsp70 to suppress the heat-induced aggregation of MDH, it indirectly showed that PfHsp70 is able to recognise and bind exposed hydrophobic surfaces on a misfolded polypeptide. Hsp70 proteins are known to recognise and bind to peptides that are eight residues long, possessing an interior hydrophobic core, which is surrounded by basic residues (Gragerov *et al.*, 1994; Jordan and McMacken, 1995). The fact that Hsp70 recognition motifs occur after an average of 36 residues along a polypeptide chain has been presented as the reason why Hsp70s tend to bind to a wide range of substrates (Rüdiger *et al.*, 1997). It is for this reason that Hsp70s bind to both short and long peptides with equal efficiency (Flynn, *et al.*, 1989).

It is known that in the ATP bound state, Hsp70 proteins have reduced affinity for substrate (Suh *et al.*, 1999). Hsp70 proteins have increased affinity for substrate in the ADP bound- and nucleotide free-state (Suh *et al.,* 1999; Revington *et al.*, 2005). Because purified PfHsp70 protein was extensively dialysed (section 5.2.6), it would be expected to be largely in a nucleotide free state. Previously, Ramya *et al.* (2006) observed that ATP enhanced the chaperone activity of PfHsp70. However, results from their study seem to contradict the fact that Hsp70 proteins have reduced substrate affinity in the presence of ATP (Suh *et al.*, 1999), since they observed 'enhanced chaperone activity' (improved substrate binding, hence a reduction in peptide substrate aggregation) when ATP was present. In contrast to this, it was expected that in the analysis conducted by Ramya *et al.* (2006), the presence of ATP in their assay would have lowered PfHsp70's affinity for peptide, promoting the heat-induced aggregation of the peptide substrate. In the present study, an MDH aggregation suppression assay (conducted in the presence of PfHsp70) was left to proceed for 27 minutes before ATP was added (Figure 5.11). The addition of ATP then triggered an increase in absorbance since ATP binding to PfHsp70 was expected to trigger the liberation of bound MDH which would subsequently aggregate due to heat. Studies demonstrating that ATP binding by Hsp70 proteins lead them to release bound peptide substrates have been carried out previously (Liberek *et al.*, 1991; Wawrzynow and Zylicz, 1995).

Work covered in this chapter involves the optimisation of PfHsp70 and PfJ4 expression in *E. coli* cells and purification by native means. Thus, the basal ATPase activity of natively purified PfHsp70 has been estimated as at least twice the value obtained in a previous study based on denatured purified protein (Matambo *et al.*, 2004). PfJ4 failed to stimulate PfHsp70 ATPase activity, suggesting that this responsibility belongs to another, yet to be identified type I or type II *P. falciparum* Hsp40. Furthermore, this study identifies the PfHsp70 arch and hydrophobic pocket residues as essential for the *in vitro* aggregation prevention function of this protein. Chapter 6 is a summary of the conclusions drawn from this study and future directions of study that could be pursued in order to further understand the chaperone role of PfHsp70 and its contribution to the physiology of *P. falciparum*.

Chapter Six

Conclusion and future work

This study established that PfHsp70 has both *in vivo* and *in vitro* chaperone properties. Structural information obtained from bioinformatic analysis indicated that PfHsp70 has conserved structural motifs for interaction with Hsp40, NEFs and substrate. Evidence confirming that this protein is heat inducible (Kumar *et al.*, 1991; Joshi *et al.*, 1992; Sharma, 1992; Biswas and Sharma, 1994), coupled to the fact this study and observations by others established that this protein has basal ATPase activity as well as the ability to inhibit protein aggregation *in vitro* (Matambo *et al.*, 2004; Ramya *et al.*, 2006) suggests that this protein plays a central role in the life cycle of proteins in *P. falciparum*.

Given the observation that PfHsp70 was able to reverse the thermosensitivity of a DnaK mutant *E. coli* strain, an interesting question is whether this was due to its role as a refoldase (facilitating the refolding of misfolded proteins back to native form) or that it merely acted as a holdase (passively inhibiting protein aggregation) or both (Slepenkov and Witt, 2002). It is known that the peptide binding domain of an Hsp70 protein on its own (in the absence of the ATPase domain) is able to recognise and bind substrates (Wu and Wang, 1999; Tanaka *et al.*, 2002; Swain *et al.*, 2006). This suggests that the presence of the peptide binding domain of an Hsp70 could be the minimum structural requirement for the suppression of protein aggregation and is able to facilitate the slow recovery of misfolded proteins to native state (Tanaka *et al.*, 2002). Based on this, it is conceivable that PfHsp70 might have been able to bind partially misfolded *E. coli* peptides, suppressing their aggregation and thus protecting the cells from heat stress, without necessarily refolding denatured proteins. However, a complete protein refolding cycle of Hsp70 requires the participation of co-chaperones (Figure 1.2). Whether PfHsp70 was able to solicit the co-operation of the *E. coli* co-chaperone machinery to enable it to operate both as a holdase and a refoldase is not certain. In fact, data emanating from the *in vivo* chaperone features of the KPf chimera (Figure 4.4) suggests that this chimera may have co-operated with *E. coli* co-chaperones as opposed to PfHsp70 which might have acted largely as a suppressor of protein aggregation.

Hsp40 proteins are regarded as 'scanning' factors for Hsp70s; sensing the presence of misfolded proteins before directing them to Hsp70s (Rüdiger *et al.*, 2001; Siegenthaler and Christen, 2006). It is therefore conceivable that in *P. falciparum*, PfHsp70 must necessarily

co-operate with an Hsp40 co-chaperone partner in order to fulfil its role as a refoldase. Indeed, the role of Hsp40 proteins as sensors in protein homeostasis has recently been highlighted by the observation that only two exposed hydrophobic patches on a particular poplypeptide are necessary in order to elicit the *cis*-transition of Hsp40 that enables it to drive ATP hydrolysis by Hsp70 (Siegenthaler and Christen, 2006). Therefore, although PfJ4 failed to stimulate the ATPase activity of PfHsp70, there could be another Hsp40 from *P. falciparum* that plays this role.

However, the fact that linker mutations (V401L and Q402K) abrogated the function of PfHsp70 *in vivo* (section 3.3.6.3) further suggests that PfHsp70 may have engaged *E. coli* co-chaperones especially DnaJ. This is because the Hsp70 linker region is essential not only for interdomain communication, but is closely connected to the regulation of the ATPase activity of Hsp70 by its Hsp40 partner (Slepenkov and Witt, 2003; Vogel *et al.*, 2006b). In addition, there are some Hsp70 residues that occur outside the linker region which regulate interdomain coupling and interaction with Hsp40 (Gässler *et al.*, 1998). Therefore, it could be argued that if PfHsp70 acted mostly through a passive holdase role, how could the linker substitutions have interfered with its function? Insight from studies conducted by Rist *et al.* (2006) showing that the linker is freely accessible to solvent in the absence of bound nucleotide and is shielded from solvent in the presence of ATP, could help answer this question. Furthermore, substrate binding through the peptide binding domain of DnaK-ATP is able to reverse the ATP-induced structural conformation, thus exposing the linker to hydration (Rist *et al.*, 2006). These findings would suggest that any changes that may abrogate the flexibility of the dynamics of the linker region could in turn interfere with peptide binding kinetics (Rist *et al.*, 2006; Vogel *et al.*, 2006b). Therefore, it is possible that the linker mutations in PfHsp70 may have interfered with its ability to bind substrates, thereby leading to its failure to protect *E. coli* DnaK mutant cells.

Another interesting observation emanating from this study was the importance of choosing *E. coli* strains carefully for use in complementation assays (Table 3.1). The three *E. coli* strains used for complementation assays displayed unique characteristics in terms of their ability to support the exogenous expression of the different proteins used in this study, consequently affecting the complementation phenotypes observed. Thus, the different features that characterise each *E. coli* strain (Table 3.1) influence the complementation results observed. For example, whilst PfHsp70 and KPf were not expressed in detectable levels in both *E. coli*

Δ*dnaK52* (Figure 3.8) and *dnaK103* (Figure 3.9) strains, functional levels of the proteins were produced in *E. coli dnaK756* strain (Figure 3.10B). Strain specific results for complementation assays have been observed in other studies (Müller *et al.*, 2000; Genevaux *et al.*, 2004).

In addition, this study suggests that PfHsp70 must be able to recognise typical Hsp70 peptide substrates (Gragerov *et al.*, 1994), since it was able to suppress protein aggregation, based on this study and work previously conducted by others (Ramya *et al.*, 2006). Interestingly, the *P. falciparum* apicoplast proteins display an abundance of Hsp70 binding sites, and mutating these residues on an apicoplast transit peptide led to the protein being mistargeted (Foth *et al.*, 2003). This indicates that one of the major roles of PfHsp70 could be in apicoplast protein trafficking (Foth *et al.*, 2003). The fact that proteins translocated into the apicoplast of *P. falciparum* possess several Hsp70 recognition sites (Foth *et al.*, 2003), begs for further study. This would establish whether PfHsp70 is involved in this process. This is important given the fact that most apicoplast enzymes are drug targets (Ralph *et al.*, 2001). In addition, PfHsp70 has been implicated in actin polymerisation (Dobrowolski *et al.*, 1997; Tardieux *et al.*, 1998), making its interaction with actin an important aspect of further study since this process is deemed to be crucial for red blood cell invasion by the parasite (Dobrowolski *et al.*, 1997).

It has been estimated that during the growth of *P. falciparum in vitro*, the parasite produces proteins so fast that in 48 hours at least 87% of total protein is constituted of newly synthesised proteins and this figure rises to at least 90% in a 60 hour growth cycle (Nirmalan *et al.*, 2004). Given the challenges that *P. falciparum* encounters in its life-cycle, it is not surprising that this organism has Hsp70 homologues that have a relative degree of structural diversity (section 2.3.2.3) in order to be able to deal with the protein folding challenges that it manages. This is important in *P. falciparum* life-cycle since the development of malaria fever adds a further strain to its protein folding machinery. The structural diversity of the Hsp70 species in *P. falciparum* observed in this study perhaps reflects the organelle specific chaperoning demands that these molecular chaperones encounter (Kappes *et al.*, 1993; Sargeant *et al.*, 2006). Although only PfHsp70 has been established as the cytosolic form of Hsp70 present in *P. falciparum*, bionformatic data strongly showed that PfHsp70-x could be an alternate cytosolic Hsp70, based on its similarity to PfHsp70 (section 2.3.2). This would

suggest a scenario where the two proteins co-operate in suppressing protein aggregation and refolding *P. falciparum* proteins that would have misfolded.

Data from the analysis of the Hsp70 protein network (Figure 5.10; LaCount *et al.*, 2005) indicate that these proteins could be involved in complex protein networks in *P. falciparum*. Although the relationship of these Hsp70s with their co-chaperones and other chaperones is not very clear from the data provided, there is indirect evidence suggesting that these proteins could interact with co-chaperones and other chaperones from *P. falciparum*. There is information suggesting that PfHsp70 could be regulated by co-chaperones through both the C-terminal EEVD motif and the N-terminal ATPase domains (Ramya *et al.*, 2006). The EEVD motif of Hsc70 protein is important for its non-competitive interaction with the co-chaperones Hop and Hsp40 (Freeman *et al.*, 1995; Demand *et al.*, 1998). It has been hypothesised that an antimalarial agent and an immunosuppressant, 15-Deoxyspergualin (DSG) interacts with Hsc70 through the EEVD motif (Nadler *et al.*, 1998). Ramya *et al.* (2006) observed that DSG was able to stimulate the chaperone activity of PfHsp70 and not that of PfHsp70-2 in the presence of ATP. Their argument was that the interaction of DSG with PfHsp70 must have been through the EEVD motif, and that because PfHsp70-3 lacks this motif, DSG could not modulate its chaperone activity. This evidence hints at the possible role of the EEVD motif in PfHsp70 in its interaction with co-chaperones. Database search on *P. falciparum* Hop homologues identified two proteins a PfHop (chr14_1.gen_156) and a p23 protein (chr14_1.gen_248) (Kumar *et al.*, 2003). Whether any of these proteins modulates the Hsp70-Hsp90 partnership (Pearl and Prodromou, 2001) is unknown. The existence of a Hop-mediated Hsp70-Hsp90 partnership in *P. falciparum* would be of interest in drug design efforts since PfHsp90 is essential for survival of the parasite (Banumathy *et al.*, 2003).

In future, some of the work that could be done includes the identification of PfHsp70's co-chaperone partner(s). Work on the investigation of PfHsp70's ability to refold misfolded peptides would help ascertain its role as a refoldase. The possible role of PfHsp70 in the trafficking of proteins into the apicoplast would be an exciting area of study. Since the apicoplast is an ideal drug design target (Ralph *et al.*, 2001), an understanding of the role of *P. falciparum* Hsp70 proteins in sustaining the metabolic pathways of this organelle would be of practical medical interest.

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Appendix A

General experimental procedures

A.1 Preparation of competent cells

A colony of *E. coli* cells was selected from a 2 x YT agar plate (2x yeast-tryptone) (1.6 g tryptone, 1.0 g yeast, 1.5 g agar, 0.5 g NaCl per 100 ml preparation in deionised water) and used to inoculate 5 ml of 2x YT broth (1.6 g tryptone, 1.0 g yeast, 0.5 g NaCl per 100 ml preparation in deionised water). The culture was allowed to grow overnight at the temperature application to the particular strain of cells. The culture was diluted in fresh 25 ml of 2x YT broth in a ratio of 1: 200 and allowed to grow to absorbance units of 0.3 -0.6 at 600 nm. The cells were harvested by centrifugating at 5 000g for 20 minutes at 4°C. The cells were then resuspended in 25 ml of ice-cold $MgCl_2$ (0.1M) and left to stand for 1 hour. The cells were resuspended in 2.5 ml of ice-cold $CaCl_2$ (0.1M). The cells were now ready for use. One volume of ice-cold 30 % glycerol was added to one volume of cells that were stored for future use at -80°C.

A.2 Transformation of competent cells

A. aliquot (100 µl) of competent cells was placed into a microcentrifuge tube in an ice bath. An alquote (2 µl) of DNA (50 ng/ µl) was added to the competent cells and gentle mixing was conducted. The cells were left on ice for 30 minutes. The cells in the microcentrifuge tube were then heat shocked at 42°C for 45 seconds and immediately placed on ice for 2 minutes. To these cells 900 µl of 2x YT broth was added, followed by incubation at the appropriate growth temperature for 1 hour with shaking. The cells were plated on 2x YT agar plates containing the appropriate antibiotics.

A.3 Small scale DNA preparation

To obtain high quality plasmid DNA preparations the Qiagen DNA minipreparation protocol (www.openwtware.org/wiki/Miniprep/Qiagen_kit) was used according to the supplier's instructions.

A.4 Agarose gel electrophoresis

To prepare 0.8 % agarose, the require amount of agarose was suspended in 0.5 x TBE buffer (45 mM Tris base, 45 mM boric acid, 1 mM EDTA pH 8.0). The agarose was heated in a microwave for approximately 60 – 90 seconds to dissolve. The agarose gel was then cooled to about 55°C before adding ethidium bromide to a final concentration of 0.5 µg/ml. The

agrose gel was the poured into the casting tank in the presence of a comb for marking the gel wells. The comb was removed, after the gel had set. The gel was placed into an electrophoresis tank and 0.5 x TBE buffer added to a level sufficient to cover the gel. Air bubbles trapped in gel wells were removed, following which the DNA was loaded. The electrophoretic tank was connected to a power source ensuring that the DNA moves towards the anode. Voltage was adjusted to 100 volts and the electrophoresis run was allowed to proceed until the gel front had travelled for about ¾ of the gel length. The DNA was then visualized and imaged using the Bio-Rad's VersaDoc™ Model 4000 imaging system

A.5 DNA restriction digestion protocol

The reagents were set up as follows:

Sterile de-ionised water	16 µl
10X restriction buffer	2 µl
DNA (100-300 ng)	2µl

The reaction was iniated by adding 2 units of restriction enzyme and the reaction allowed to proceed for at least 1 hour at 37°C or as recommended. To stop the reaction, 4 µl of gel loading buffer (0.25 % bromophenol blue + 30 % glycerol) was added.

A.6 Site-directed mutagenesis using Quik-change (Strategene) protocol

Site-directed mutagenesis was carried out using the Quik-change kit (Strategene, U.S.A) according to the supplier's protocol. The reagents were set up as follows:

Control reaction

Reagent	**Volume (µl)**
Sterile deionised	39.5
10 x reaction buffer	5
pWhitescript 4.5-kb control plasmid (5 ng/µl)	2
Oligonucleotide primer #1 (125 ng/µl)	1.25
Oligonucleotide primer #2 (125 ng/µl)	1.25
dNTP mix	1
Total volume	**50**

Then 1 µl of *PfuTurbo* DNA polymerase (2.5 U/µl) is added

Test reaction

Reagent	**Volume (µl)**
Sterile de-ionised water	40
10 x buffer	5
dNTP mix	1
Forward primer (125ng/µl)	1
Reverse primer (125ng/µl)	1
Template DNA (100ng/µl)	2
Total volume	**50**

Then 1 µl of *PfuTurbo* DNA polymerase (2.5 U/µl) is added

The reaction was then conducted under the following cycling conditions (Table A.1):

Table A.1 Cycling parameters for site-directed mutagenesis

Segment	No of cycles	Temperature	Time
1	1	95°C	30 seconds
2	12-18	95°C	30 seconds
		55°C	1 minute
		68°C	1minute/kb of plasmid length

After the cycling, the reaction is allowed to cool before 1 µl of the *Dpn* I restriction enzyme (10 U/µl) is added to each reaction tube. The tubes are incubated at 37°C for 1 hour, and the products analysed by agarose gel elctrophoresis; loading 10 µl of each product into the gel.

A.7 DNA gel purification from gel bands using the Amersham (U.S.A) kit protocol

The following is a summary of the steps used for DNA purification from an agarose gel

- About 10 mg of gel slice was cut using a razor blade
- The gel slice was weighed and placed in a 1.5 ml microcentrifuge tube
- Capture buffer (10 µl) was added, the tube was closed and vortexed vigorously, followed by incubation at 60°C to dissolve the agarose compeletely
- Agarose was centrifuged briefly and transferred into the GFX column
- The product was left to sit in the column for 1 minute, followed by centrifugation at full speed for 1 minute
- Flow through was discarded, and column was placed back in the collection tube
- Wash buffer (500 µl) was added, followed by centrifugation 30 seconds
- The collection was discarded and column was transferred to a fresh collection tube

- Using an aliquot of water (50 µl) or T.E buffer (Tris-cl; pH 8.0), the DNA was eluted by centrifugation at full speed for 1 minute.

A.8 Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE)

The resolution of proteins was carried out using 12 % acrylamid resolving gel using the concept of staggered buffering system (Laemmli, 1970). The electrophoresis was conducted using the Bio-Rad Mini protein 3 electrophoresis system (Biorad, U.S.A). Preparation of resolving and stacking buffers was carried out as shown (Table A3.4). Gels were allowed to stand for 30 minutes to the polymerisation to complete. They were then transferred into the electrophoresis tank and electrophoresis buffer (25 mM Tris, pH 8.3 250 mM glycine and 0.1 % (w/v) SDS) was added.

In order to protein samples samples for analysis, they were mixed with SDS sample buffer (0.25 % Coomasie Brilliant blue (R250); 2 % SDS; 10 % glycerol (v/v); 100 mM tris; 1 % mercaptoethanol) in a ratio of 1 : 1 and boiled for 5 minutes at 95°C. The protein samples, were loaded into gel wells. Bio-Rad premixed molecular weight markers were also loaded. The electrophoresis was allowed to run for at least 1 hour at 150 volts.

Table A.2 Solutions for making a 5 % stacking gel and a 12 % resolving gel for SDS-PAGE

Reagent (ml)	5 % stacking gel	12 % resolving gel
Distilled water	4.1	4.9
30 % polyacrylamide	1.0	6.0
1.5 M Tris (pH 8.8)	-	3.8
1.0 M Tris (pH 6.8)	0.75	-
10 % Ammonium persulphate	0.06	0.15
TEMED	0.006	0.006
Total volume	6 ml	15 ml

A.9 Western blotting analysis of proteins

SDS-PAGE gels were removed from glass plates after the electrophoresis and the stacking gel cut off. The following were immersed in transfer buffer (25 mM Tris; 192 mM glycine

and 20 % methanol) to equilibrate for about 30 minutes at 8°C: gel; 3 mm Whatman filter papers, two Scotchbrite fibre pads and nitrocellulose western transfer paper (Hybond-C extra, ECL; Amersham, U.S.A).To prepare the gel for transfer, the gel was placed on the filter paper on top of the Scotchbrite pad, ensuring that air bubbles were not trapped. The nitrocellulose paper was then laid over the gel , followed by the filter paper and the other Scotchbrite pad. The transfer sandwich was placed in the transfer holder in such a way that proteins will migrate towards the nitrocellulose membrane. The transfer was run for 1 hour at 100 volts, ensuring that the transfer system was kept cool by placing a heat trap in the transfer buffer. The success of the transfer process was confirmed by staining the blot with the Ponceau stain. To visualize the bands, chemilunescence based Western anlysis was employed.

A.10 Chemiluminescence-based visualisation of Western blots

The production of the His-tagged proteins and DnaK was confirmed using anti-His antibodies (Amersham, U.S.A) and monoclonal antibodies specific for *E. coli* DnaK (Stressgen Biotechnology, U.S.A) respectively. Chemiluminscence-based immunodetection was performed using the ECL kit from Amersham (U.S.A), following the supplier's instructions with minor modifications. The membrane was blocked using blocking solution (5 % non-fat milk in TBS). Blocking was allowed to proceed for 1 hour. The membrane was briefly washed in TBS, followed by incubation with the primary antibody for 1 hour. The membrane was washed three times using TBS-Tween (TBS + 0.1 % tween 20) for 15 minutes each wash. The membrane was then incubated with the secondary antibody for 1 hour. The membrane was washed at least 3 times using TBS-Tween. The Western blots were developed for visualisation using the ECL Chemiluminescent kit, following the supplier's instructions. The Western blots were then visualised using the Bio-Rad's VersaDoc™ Model 4000 imaging system.

A.11 DNA sequencing protocol

The reactions for thermocycling were conducted by preparing 250 – 500 ng of pure DNA mixed with 3.2 pmol of primer. The total volume of DNA and primer was made up to 12 µl using triple distilled water. To this mixture, 8 µl of BigDye V3.1 (ABI, U.S.A) was added prior to the amplification. The ampilification was carried out as follows: rapid thermal ramp to 96°C, the temperature was held at 96°C for 10 seconds, followed by thermal ramp at 50°C and this temperature was held for 5 seconds. This was followed by a rapid thermal ramp to

60 °C, which was maintained for 4 minutes. The temperature was dropped rapidly to 4 °C and the product held at this temperature for collection. The product was then cleaned using the DNA Clean and Concentrator-5 columns (Zymo Research, California, U.S.A), according to the supplier's instructions. The product was vacuum dried before sequencing. DNA sequencing was carried out based on the chain termination method. The ABI 3100 Genetic Analyser (ABI, U.S.A), with a capillary electrophoresis based system was used for sequencing.

Appendix B

Primers used for site-directed mutagenesis

Table B.1 Generation of modified plasmids used for the construction of chimeric proteins

Modified plasmid	Codon changes	Forward primer used	Reason for mutation	Diagnostic restriction enzyme
pQE30/PfHsp70-EL	gcc: gca	caaaaaatagtagagc**a**ttaagaagat taagaa	Elimination of *Afl* II restriction site	*Afl* II
pQE30/PfHsp70-V401L/Q402K	gtc : ctt caa : aag	caatcaaatgct**ctt**a**a**ggatttattatta ttag	Introduction of *Afl* II restriction site	*Afl* II
pQE30/PfHsp70-V401L	aag : caa	ggtgaccaatcaaatgctctt**caa**gatt tattattattag	PfHsp70-401L/Q402K to PfHsp70-V401L change	*Afl* II
pQE30/PfHsp70-Q402K	ctt : gtc	ggtgaccaatcaaatgct**gtc**aaggat ttattattattag	PfHsp70-401L/Q402K to PfHsp70-Q402K change	*Afl* II
pQE60/DnaK-V386L	gta : ctt aaa : aag	ctgactggtgac**ctt**a**a**ggacgtactg ctg	Introduction of *Afl* II restriction site	*Afl* II
pQE30/PfK	ctt : gtc aag : caa	ggtgaccaatcaaatgct**gtc**c**aa**gac gtactgctgc	PfK-V401L/Q402K to PfK	*Afl* II

Codon changes introduced on the primers are highlighted in bold.

Table B.2 Primers used for generation of modified plasmids forPfHsp70 substrate binding cavity studies

Modified plasmids	Codon changes	Forward primer used
pQE30/PfHsp70-A419Y and pQE60/KPf-A404Y	gaa : gag act : aca gct : tat	cattaggtttaga**gac**a**tat**ggtggtgttatg
pQE30/PfHsp70-Y444A and pQE60/KPf-Y429A	tat : gca	ctttactact**gca**gctgataaccaacc
pQE30/PfHsp70-V451F and pQE60/KPf-V436F	gtc : ttt	ccaaccaggttttttaattcaagtatatg

Codon changes introduced on the primers are highlighted in bold.

Table B.3 Primers used in the convert PfJ4-D80F/N182S to PfJ4 by site directed mutagensis

Name of primer	**Codon changes**	**Amino acid change**	**Forward primer**	**Diagnostic endonuclease**
J4 F80D Fw	ttc : gac	F to D	cgaaaactacatggcggacgaaaac**ga**cgaattc	*Bst* BI
J4 S182N	tcg : aac	S to N	ctggcaagttcaag**aac**cgagttgttaaaacc	*Nru* I

Codon changes introduced on the primers are highlighted in bold.

Appendix C

Phosphate standard curve

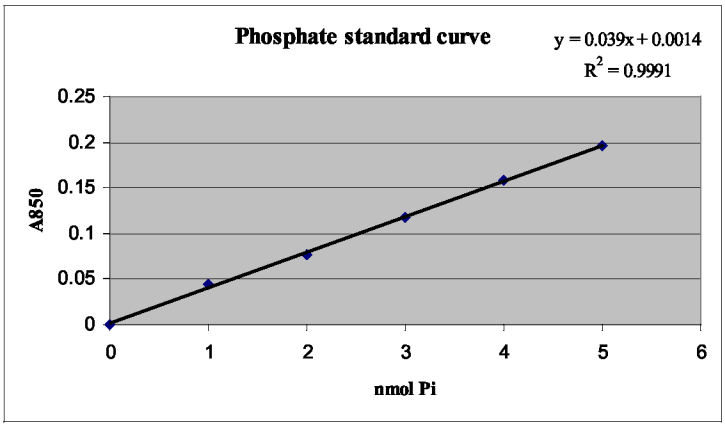

Figure C.1 Phosphate standard curve
The plot shows the absorbance values of standard samples containing 0-5 nmol of KH_2PO_4 determined by the colourimetric assay using a UV/Vis-spectrophotometer. The standard curve was produced in order to determine the ATPase activity of PfHsp70 by measuring rate of Pi release.

Appendix D

Special reagents and chemicals

Table D.1 Special chemical reagents and kits

Name of reagent	Vendor/supplier
Agarose	Whitehead scientific, South Africa
Acetic acid	Merck, Germany
Ammonium molybdate	Merck, Germany
Ammonium persulphate	Merck, Germany
Ampicillin	Roche, Germany
Ascorbic acid	Merck, Germany
Adenosine triphosphate	Roche, Germany
Chemiluminescence Western blotting kit	Amersham, U.S.A
β-mercaptoethanol	Merck, Germany
Bovine serum albumin	Roche, Germany
Bromophenol blue	Sigma, U.S.A
Calcium chloride	Meck, Germany
Coomasie brilliant blue R250	Amersham, U.S.A
Chloramphenicol	Roche, Germany
Diethiothreitol	Roche, Germany
dNTP mix	Roche, Germany
Ethidium bromide	Sigma, U.S.A

Glacial acetic acid	Merck, Germany
Glycerol	Merck, Germany
Glycine	Sigma, U.S.A
HEPES	Amersham, U.S.A
Hybond C-extra	Amersham, U.S.A
Imidazole	Sigma, U.S.A
Isopropyl-1-thio- β-D-galacopyranoside	Roche, Germany
Potassium chloride	Merck, Germany
Potassium dihydrogen phosphate	Merck, Germany
Lambda DNA	Promega, U.S.A
Lysozyme	Roche, Germany
Methanol	Merck, Germany
Magnesium chloride	Merck, Germany
Monoclonal mouse anti-His antibody	Amersham, U.S.A
N-laurylsarcosine	Sigma, U.S.A
Ni-chelating sepharose	Amersham, U.S.A
Phenylmethylsufonyl fluoride	Sigma, U.S.A
Polyacrylamide	Sigma, U.S.A
Polyethylene glycol 2 000	Merck, Germany
Polyethylenimine	Sigma, U.S.A
Ponceau S	Amersham, U.S.A
Rapid ligation buffer	Promega, Germany
Restriction enzymes	New England Biolabs, U.S.A
Sodium chloride	Merck, Germany
Sodium dodecyl sulphate	Merck, Germany
Sodium hydroxide	Merck, Germany
Snakeskin™ pleated dialysis tubing	Pierce, U.S.A
TEMED	Sigma, U.S.A
Tris	Sigma, U.S.A
Tryptone	Oxoid, U.K
Tween 20	Merck, Germany
Urea	Promega, Germany
Yeast	Oxoid, U.K