BIOCHEMICAL CHARACTERIZATION OF *PLASMODIUM FALCIPARUM* HEAT SHOCK PROTEIN 70

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Biochemical characterization of the Plasmodium falcipaum Hsp70

ABSTRACT

Plamodium falciparum heat shock protein (PfHsp70) is believed to be involved in the cytoprotection of the malaria parasite through its action as a molecular chaperone. Bioinformatic analysis reveal that PfHsp70 consists of the three canonical Hsp70 domains; an ATPase domain of 45 kDa, Substrate binding domain of 15 kDa and a C-terminal domain of 10 kDa. At the C-terminus there is a GGMP repeat motif that is commonly found in Hsp70s of parasitic origins. *Plasmodium falciparum* genome is 80% A-T rich, making it difficult to recombinantly express its proteins in *Escherhia coli* (*E. coli)* as a result of rare codon usage. In this study we carried out experiments to improve expression in *E. coli* by inserting the PfHsp70 coding region into the pQE30 expression vector. However multiple bands were detected by Western analysis, probably due to the presence of rare codons. The RIG plasmid, which encodes tRNAs for rare codons in particular Arg (AGA/AGG), Ile (AUA) and Gly (GGA) was engineered into the *E. coli* strain resulting in production of full length PfHsp70. Purification was achieved through Ni^{2+} Chelating sepharose under denaturing conditions. PfHsp70 was found to have a very low basal ATPase activity of 0.262 ± 0.05 nmoles/min/mg of protein. In the presence of reduced and carboxymethylated lactalbumin (RCMLA) a 11-fold increase in ATPase activity was noted whereas in the presence of both RCMLA and *Trypanosoma cruzi* DnaJ (Tcj2) a 16-fold was achieved. For ATP hydrolysis k_{cat} value of 0.003 min^{-1} was obtained whereas for ADP release a greater k_{cat} value of 0.8 min^{-1} was obtained. These results indicated that rate of ATP hydrolysis maybe the rate-determining step in the ATPase cycle of PfHsp70.

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LIST OF ABBREVIATIONS

EDTA	**:**	Ethylenediamine tetra-acetic acid
His-tagged	**:**	Histidine – tagged
IPTG	**:**	Isopropy-1-thio-beta-D-galactopyranoside
mSTI1	**:**	murine stress –inducible protein1
PBS	**:**	Phosphate buffer saline
PMSF	**:**	Phenylmthylsulfonyl Flouride
SDS	**:**	Sodium dodecylsulphate
SDS-PAGE	**:**	SDS-polyacrylamide gel electrophoresis
TBS	**:**	Tris buffer saline
TBST	**:**	TBS-Tween
Tris	**:**	Tris (hydroxymethyl) aminomethane
2x YT	**:**	2xYeast-Tryptone
Hsc 70	**:**	Heat shock cognate 70
Hsp70	**:**	Heat shock protein 70
Hsp40	**:**	Heat shock protein 40
Hsp90	**:**	Heat shock protein 90
PfHsp70	**:**	*Plasmidum Falciparum* Heat shock protein 70
BovHsc70	**:**	Bovine Heat shock cognate 70
TcHsp70	**:**	*Trypanosoma cruzi* Heat Shock protein 70
Hip	**:**	Hsc70-interacting protein
Hop	**:**	Hsc70-Hsp90 organizing protein
GGMP	**:**	Glycine-Glycine-Methionine-Proline
EEVD	**:**	Glutamate-Glutamate-Valine-Asparatate
Ni-NTA	**:**	Nickel-nitrilotriacetic acid
Pi	**:**	Inorganic phosphate
KCl	**:**	Potassium chloride
MgCl	**:**	Magnesium Chloride
ATP	**:**	Adenosin Triphosphate
ADP	**:**	Adenosin diphosphate
H_2SO_4	**:**	sulphuric acid
Na_2HPO_4	**:**	Sodium Hydrogen orthophosphate
HEPES	**:**	N-(2-hydroxyethyl)piperrazine-N’-(2-ethanesulfonic acid)

DNTP	**:**	Deoxyribonucleoside Triphosphate
DTT	**:**	Dithiothreitol
$(NH_4)_2S_2O_8$	**:**	Ammonium persulphate
NaCl	**:**	Sodium Chloride
RIL	**:**	Arginine, Isoleucine, and Leucine
RIG	**:**	Arginine, Isoleucine, and Glycine
HPD	**:**	Histidine, Proline and Aspartate
RCMLA	**:**	reduced and carboxymethylated lactalubumin
FPLC	**:**	Fast Performance Liquid Chromatography
HPLC	**:**	High Performance Liquid Chromatography

CHAPTER 4

IN VITRO CHARACTERIZATION OF CHAPERONE ACTIVITY OF PfHsp70

4.1 INTRODUCTION

4.1.1 ATPase ACTIVITY

In eukaryotes several cofactors have been identified which associate and cooperate with Hsc70 (Luders *et al*., 1998). Association with cofactors is essential for the regulation of the ATPase cycle of Hsc70. Altering the affinity of the chaperone for nonnative polypeptide substrates. Hsc70 in the ATP-bound conformation rapidly binds and releases a polypeptide substrate; stable substrate binding depends on the transfer of the chaperone to the ADP-bound state. ATP hydrolysis by Hsc70 is stimulated by cofactors of the Hsp40 protein family, such as Hsp40 (Hdj-1, Hdj-2, and auxilin). Hsp40 is thus required for efficient substrate recognition by Hsc70 ADP-bound state of Hsc70 generated through an interaction with Hsp40, is affected by the Hsc70 cofactors Hip (Hsp70-interacting protein) and BAG-1 in opposite ways. Hip slows down the release of ADP from Hsc70 and may therefore stabilize an Hsc70/substrate complex. BAG-1 accelerates ADP release from the chaperone protein, thus strongly stimulating Hsc70's ATPase activity in conjunction with Hsp40. (Hip) and BAG-1 both bind to the ATPase domain of Hsc70 in a mutually exclusive manner. Hip and BAG-1 represent competing Hsc70 cofactors that conversely affect the ADP-bound state of the chaperone protein (Luders *et al*., 1998).

In studying the complex formation of Hsp70 with peptide, reduced and carboxymethylated lactalbumin (RCMLA) may be used. RCMLA, which in its native form resembles an unfolded protein interacts with Hsp70 under non-denaturing conditions (Palleros e*t a.,l* 1991).

4.1.2 OBJECTIVE

To date no work has been carried out on the chaperone activity of PfHsp70 therefore the nature of the chaperone activity of PfHsp70 is not known. The broad objectives of this study were to investigate any possible chaperone activity of PfHsp70 by carrying out basal ATP hydrolysis by measuring release of inorganic phosphate. This was done by stimulating the ATP hydrolysis in the presence of cochaperone and substrate, and also determining the rate limiting steps that occur during ATP hydrolysis and ADP release.

4.2 EXPERIMENTAL METHODOLOGY

4.2.1 MATERIALS

All reagents were obtained from Sigma Chemicals Co. (St.Louis,Mo U.S.A.), Roche Molecular Biochemicals (Indianapolis, IN, U.S.A.) or Merck Chemicals (Darmstadt, Germany) unless stated otherwise. pET28a/Tcj2 was generated by Dr.D.M. Engman (Department of Pathology, Northwestern University Medical school, USA). L-Lactic Dehydrogenase (type XI; from Rabbit muscle, Sigma, U.S.A.), Pyruvate kinase (type II; from Rabbit muscle, Sigma, U.S.A.), Phosphoenolpyruvate (PEP, Roche, U.S.A.), NADH (Roche, U.S.A.)

4.2.2 ATPase ACTIVITY OF PfHsp70

4.2.2.1 BASAL ATP HYDROLYSIS BY MEASURING RELEASE OF INORGANIC PHOSPHATE

Serial dilutions of 1 mM Na_2HPO_4 of concentrations randging from 0 to 300 μM were prepared. A solution of 1.25 % Ammonium molybdate in 6.5 %H_2SO_4 (1 ml) and 9 % Ascorbic acid (1 ml) were added and the reactions incubated at room temperature for 30 minutes before reading the absorbance at 660 nm.

Reaction mixtures were prepared (Appendix A6, Table A3). PfHsp70 was purified as before in chapter 3. The enzymes were pre-incubated at 37˚C for 5 minutes in the buffer. Reactions were started by addition of ATP. The reactions were incubated for 30 minutes and samples were taken every 2 minutes for 10 minutes, and from then at 10 minute intervals. Sample (50 μl) was collected and the reaction was stopped by the addition of 50 μl 10 % SDS. 1.25 % ammonium molybdate in 6.5 % H_2SO_4 (50 μl) and 9 % ascorbic acid (50 μl) were added to develop the colour. The reaction was incubated at room temperature for 30 minutes, and absorbance of the bluish-purple colour was read at 660 nm. Boiled PfHsp70 control was included.

4.2.2.2 Stimulation with substrate (RCMLA)

The ATP hydrolysis assay was carried out as in section 4.2.2.1. Modifications made were incubating 0.4 μM of Pfhsp70 with final concentrations of 0.1 μM, 0.4 μM, and

1 μM of RCMLA for 5 minutes before starting reactions with the addition of 4 mM ATP.

4.2.2.3 Co-chaperone (Tcj2) purification

Clear lysate was added to Ni-charged chelating sepharose resin (1 ml). The mixture was gently agitated 4 ºC for 2 hours, after which the resin was loaded in a 10 mL syringe, plugged with glass wool. The flow through was collected and the resin washed three times with Native Wash Buffer (300 mM NaCl, 150 mM imidazole, 0.01 M Tris, pH 8.0). Proteins bound to the resin were eluted three times with Native Elution buffer (300 mM NaCl, 1 M imidazole, 0.01 M Tris, pH 8.0). Purified proteins were stored at –70 ºC until further use.

4.2.2.4 Stimulation with co-chaperone

The ATPase assay was carried out as in section 4.2.2.1. Modifications made were incubating 0.4 μM Pfhsp70 with final concentrations of 0.1, 0.4, 1 μM of Tcj2 for 5 minutes before starting reactions with addition of ATP. In the presence of substrate (RCMLA), PfHsp70 was incubated with Tcj2 and substrate for 5 minutes before starting reactions with the addition of ATP.

4.2.3 MECHANISTIC STUDIES OF RATE LIMITING STEP

4.2.3.1 DETERMINATION OF V_{max}, AND K_m FOR ATP HYDROLYSIS

Eight ATP hydrolysis reactions were prepared (see Appendix A6, Table A3). PfHsp70 was added to a final concentration of 0.4 μM, and the volume made up to 400 μl with ultrapure distilled water ($dddH_20$). The enzymes were pre-incubated at 37˚C for 5 minutes in the buffer. Reactions were started by adding varying concentrations of ATP 0 to 1000 μM respectively, incubated for 30 minutes, and samples taken every 2 minutes for 10 minutes, and from then every 10 minutes interval. Samples were collected (50 μl), and the reaction was stopped by the addition

of 50 µl 10 % SDS. A solution of 1.25 % ammonium molybdate in 6.5 % H_2SO_4 (50 µl) and 9 % ascorbic acid (50 µl) were added to develop the colour. The reaction was incubated at room temperature for 30 minutes, and absorbance of the bluish-purple colour was read at 660 nm.

4.2.3.2 V_{max}, K_m AND k_{cat} FOR ADP RELEASE

To eight 1 ml reactions the following reagents were added; 0.482 units of L-Lactic Dehydrogenase (type XI; from Rabbit muscle,Sigma,U.S.A.), 0.784 units of Pyruvate kinase (type II; from Rabbit muscle, Sigma,U.S.A.), 1.6 mM of Phosphoenolpyruvate (PEP,Roche,U.S.A.), and 4 mM ATP to final concentrations between of 0 to 1000 µM of NADH (Roche,U.S.A.) in buffer containing (75 mM Potassium acetate and 2 mM $MgCl_2$). Reactions were started by the addition of 0.4 µM of PfHsp70. Activity was monitored at 340 nm as a function of the disappearance of NADH (Valero *et al.*, 2000). Two controls were included; one containing no ATP and the other containing no NADH.

4.3 RESULTS AND DISCUSSION

4.3.1 BASAL ATP HYDROLYSIS BY MEASURING RELEASE OF INORGANIC PHOSPHATE

The basal ATP hydrolysis activity of PfHsp70 was calculated to be 0.262 ± 0.05 nmoles/min/mg of protein (Table 4.1). It was found to be 4 times lower than that of Human Hsc70 with an activity of value of 1.08 ± 0.05 nmoles/min/mg of protein (Chamberlain and Burgoyne 1997). However when the ATPase activity of Human Hsc70 was calculated using our protocol it was found to have an activity of 2.06 ± 0.07 nmoles/min/mg of protein (Table 4.1). PfHsp70 was also found to be 2 times lower than that of Human Hsp70 (0.50 ± 0.30 nmoles/min/mg of protein) (Chamberlain and Burgoyne 1997). This low ATPase activity may reveal that substrates and co-chaperones (*Plasmodium falcipaurm* DnaJ proteins) may be required for full stimulation of the ATP hydrolysis activity of PfHsp70. PfHsp70 was denatured by boiling at 100ºC for 10 min, and as expected no activity was observed (Figure 4.1).

Table 4.1 Specific enzyme activities of Hsc70, Hsp70 and PfHsp70

Protein	ATPase activity (nmoles/min/mg of protein)
*Hsc70	1.08 ± 0.05
†Hsc70	2.06 ± 0.07
*Hsp70	0.50 ± 0.30
PfHsp70	**0.262 ± 0.05**

* Reported activities for Hsc70 and Hsp70 (Chamberlain and Burgoyne, 1997)

†Hsc70 – Calculated activity (protocol used to calculate PfHsp70)

± Represents the standard deviation

4.3.2 STIMULATION WITH SUBSTRATE (RCMLA)

Reduced and carboxymethylated lactalbumin (RCMLA), is a protein that resembles an unfolded protein in its native conformation (Palleros *et al.*, 1991). Basal ATP hydrolysis activity of PfHsp70 was stimulated in the presence of RCMLA (Figure

4.1). This stimulation suggested an association probably occurred between PfHsp70 and RCMLA resulting in an increase in ATP hydrolysis activity. Figure 3.8 (Chapter 3) supports the probability of an association. The highest activity was noted when using 6 μM of RCMLA; this revealed an increase in activity by 11- fold of 2.89 nmoles/min/mg of protein (Figure 4.1).

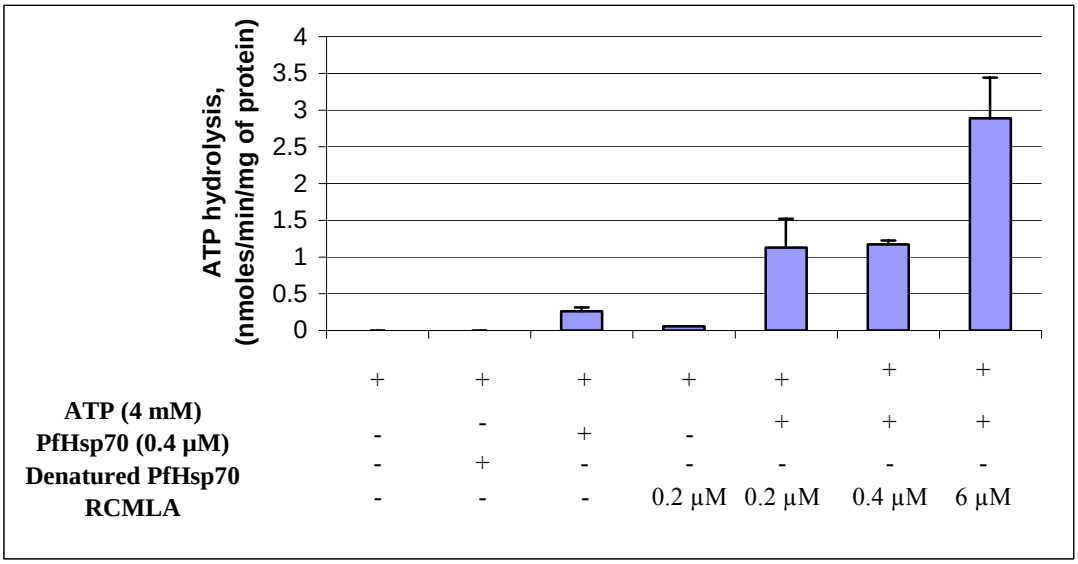


Figure 4.1: Effects of substrate on the ATP hydrolysis activity of PfHsp70.

Protein concentrations used were: PfHsp70, 0.4 μM; RCMLA (0.2 μM, 0.4 μM, 0.6 μM and 6 μM). In the presence of RCMLA the activity increased as the concentration of RCMLA increased, with the presence of 6 μM RCMLA stimulating an 11-fold increase.

4.3.3 CO-CHAPERONE (Tcj2) PURIFICATION

Very little work has been carried out on the DnaJ proteins of *Plasmodium falciparum* (Pfj 1-4). At the time of experiments Pfj proteins were not available, therefore a heterologous system was performed using *Trypanosoma cruzi* DnaJ 2 (Tcj 2) as co-chaperone, as this is a parasitic DnaJ protein. A multiple sequence alignment revealed that both Tcj 2 and Pfj 1 are Type I DnaJ proteins; these are characterized by an N-terminal J-domain, followed by a glycine/phenylalanine (G/F) rich region and a CysxxCysxGlyxGly region (Figures 2.5 and 2.6) (Chapter 2). $(His)_6$-Tcj2 was purified using Ni-chelating affinity under native conditions (Figure 4.2).

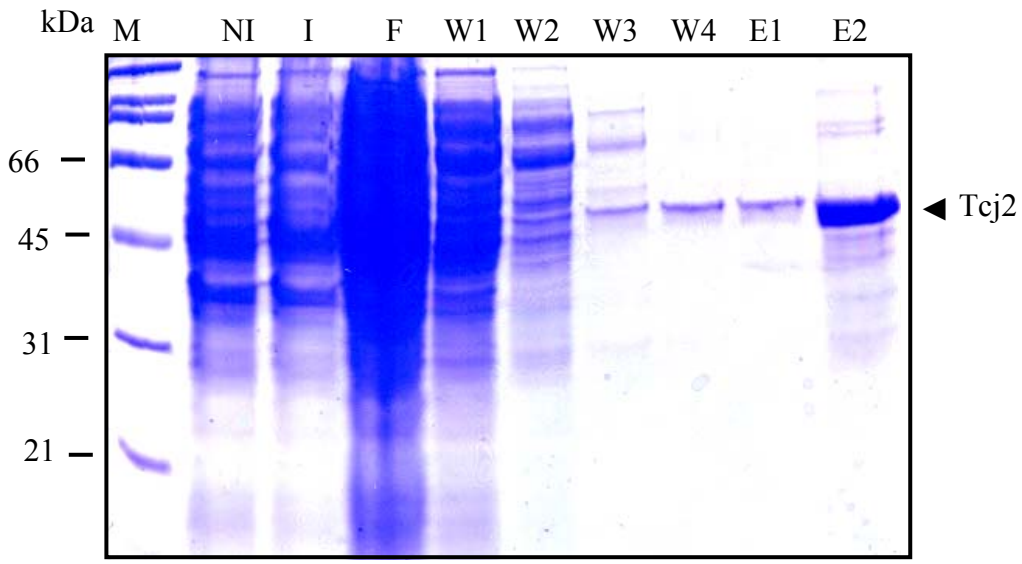


Figure 4.2: Ni-affinity Purification of $(His)_6$-Tag Tcj2.

Lane M: Molecular mass markers, Lane NI: Non induced whole cells, Lane I: Induction of Whole cells after 2 hours, Lane F: Flow through, Lanes W1-4: Washes 1-4, Lanes E1-2: Elutions 1-2 using 1 M imidazole.

4.3.4 STIMULATION WITH CO-CHAPERONE

ATPase activity carried out in the presence of 0.1 μM of Tcj2 showed an increase of 15-fold giving an activity of 4.08 nmoles/min/mg of protein. A 10-fold increase in activity to 2.62 nmoles/min/mg of protein was achieved with 0.4 μM of Tcj2 but using 1 mM of Tcj2 no activity was recorded (Figure 4.3), however Work carried out by Minami *et al* (1996) showed that Hsp 40 stimulated Hsc 70 by 7-fold. In the presence of 0.1 μM of Tcj2 and RCMLA (6 μM) there was a 16-fold increase in activity givig an activity of 4.3 nmoles/min/mg of protein, but in the presence of 0.4 μM of Tcj2 and RCMLA (6 μM) there was only a 11-fold increase and no activity was noted with 1 mM Tcj2 (Figure 4.3). It was noted that Both Tcj2 and RCMLA were able to stimulate PfHsp70. An increase in activity was noted using sub molar concentrations of Tcj2 in the presence and absence of RCMLA. However as Tcj2

concentration was increased activity decreased decreased it was noted that in the presence of 1 mM Tcj2 there was no ATPase activity recorded in the presence of RCMLA. This suggested that high levels of Tcj 2 may block the ATP/ADP binding site.

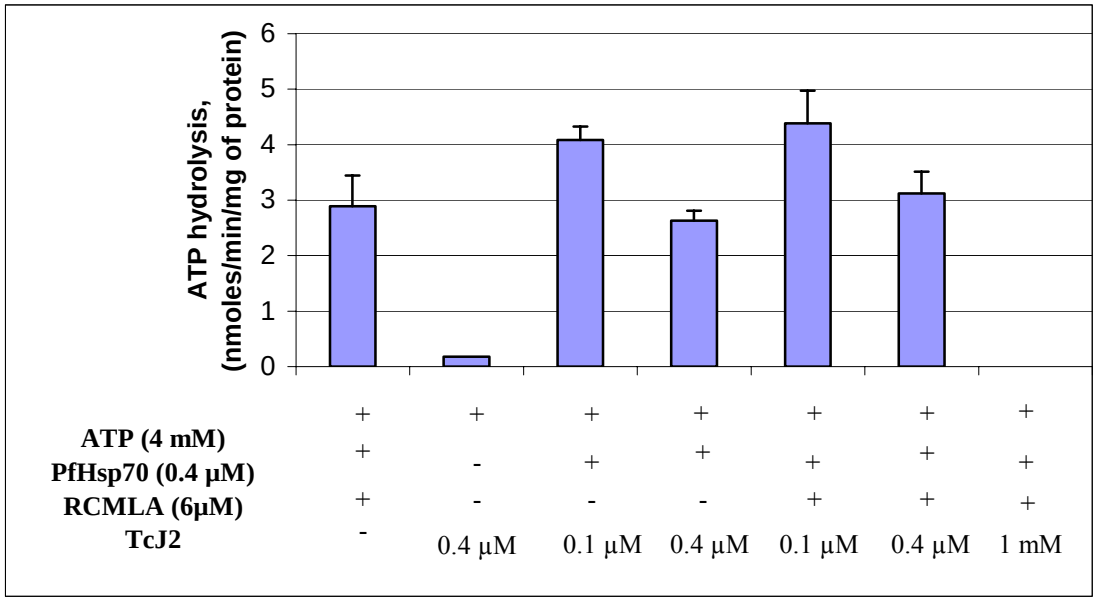


Figure 4.3: Effect of co-chaperone upon the basal ATP hydrolysis activity of PfHsp70.

Protein concentration in the assays were: PfHsp70, 0.4 µM; RCMLA, 6 µM and Tcj2, 0.1 µM and 0.4 µM. In the presence of a co-chaperone (Tcj2) there was a further ATPase activity increase by 16-fold using 0.1 µM (Tcj2) and 11-fold increase using 0.4 µM (Tcj2). As Tcj2 concentration was increased the ATPase activity decreased.

4.3.5 MECHANISTIC STUDIES: RATE LIMITING STEP OF PfHsp 70 ATPase CYCLE

4.3.5.1 V_{max}, K_m and k_{cat} of ATP HYDROLYSIS

In determining the V_{max} of PfHsp70, 0.4 µM of PfHsp70 was used in the presence of varying concentrations (20-400 µM) of ATP (Figure 4.4). A Lineweaver-Burk plot was used to calculate the $V_{max,}$ K_m and k_{cat} values of PfHsp70. PfHsp70 exhibited a high V_{max} value of 0.916 nmoles/min and a high K_m of 395.940 µM, and a low k_{cat} value of 0.003 min^{-1}. The k_{cat} values of PfHsp70 was low compared to that of Hsp70 (0.14 min^{-1}) and *E.coli* DnaK (0.03 min^{-1}) (Bimston *et al.,* 1998), (Kamath-Loeb *et*

al., 1995) (Table 4.2). k_{cat} values were determined as in equation 1 where V_{max} is the maximum velocity and $[E]_t$ is the total enzyme concentration.

$$k_{cat} = V_{max}/[E]_t \qquad (1)$$

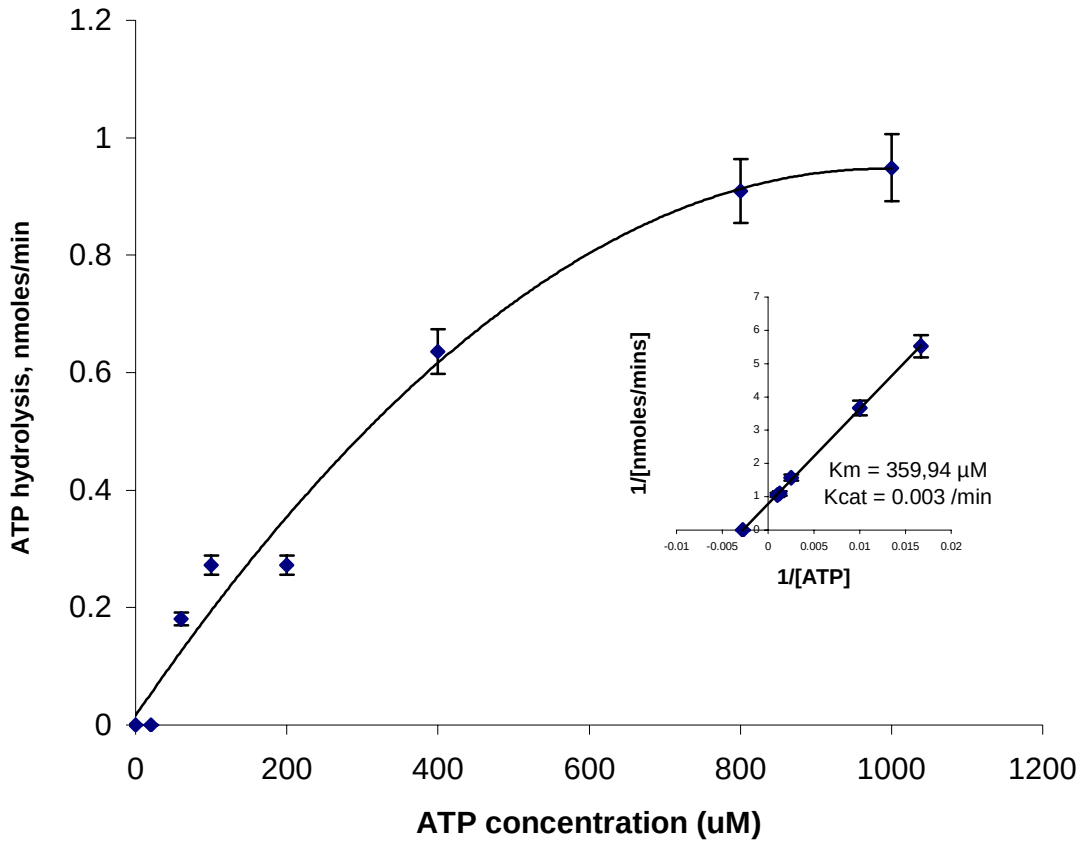


Figure 4.4: ATP hydrolysis activity of PfHsp70 as a function of ATP concentration.

0.4 µM of PfHsp70 was used to determine the optimum concentration of substrate (ATP). Michaels-Menten plot was produced. In the insert the Lineweaver-Burk plot was also produced. The V_{max}, K_m and K_{cat} were determined using these plots. (**V_{max} 0.916 nmoles/min, K_m 359.940 µM and K_{cat} 0.003 min $^{-1}$**

Table 4.2 Turnover rates (kcat) repoted for various Hsp70s

Protein	**K_{cat} (min^{-1})**
*Hsp70	0.14
*Dnak	0.03
PfHsp70	**0.003**

*Reported Hsp70 and DnaK (Bimston et al., 1998)(Kamath-Loeb et al., 1995)

4.3.5.2 V_{max}, K_m AND k_{cat} FOR ADP RELEASE

The K_{cat} for ADP release was achieved by calculating the amount of NADH reduced. This reaction was carried out using NADH as the substrate. The disappearance of NADH by conversion to NAD^+ was monitored at 340 nm; equations 2 and 3 describe the mechanism. ADP is produced during hydrolysis of ATP and it is released and reacts with PEP in the presence of Pyruvate kinase (PK) to produce Pyruvate and ATP (equation 2). Pyruvate further reacts with NADH in the presence of L-Lactic Dehydrogenase (LDH) to produce NAD^+ and Lactic acid (equation 3). The rate at which ADP is released from the ATP/ADP cleft is measured by the rate at which NADH is converted to NAD^+. The value obtained for k_{cat} was found to be 0.806 min^{-1} (Figure 4.5). The k_{cat} value obtained for ADP release was compared to that of ATP hydrolysis, these values gave an indication of the rate-determining step. ADP release had a greater k_{cat} value than that of ATP hydrolysis, suggesting that ATP hydrolysis may be the rate-limiting step in ATPase activity of PfHsp70.

$$\text{ADP} + \text{PEP} \xrightarrow{\text{PK}} \text{Pyr} + \text{ATP} \qquad (2)$$

$$\text{Pyr} + \text{NADH} \xrightarrow{\text{LDH}} \text{NAD}^+ + \text{Lac} \qquad (3)$$

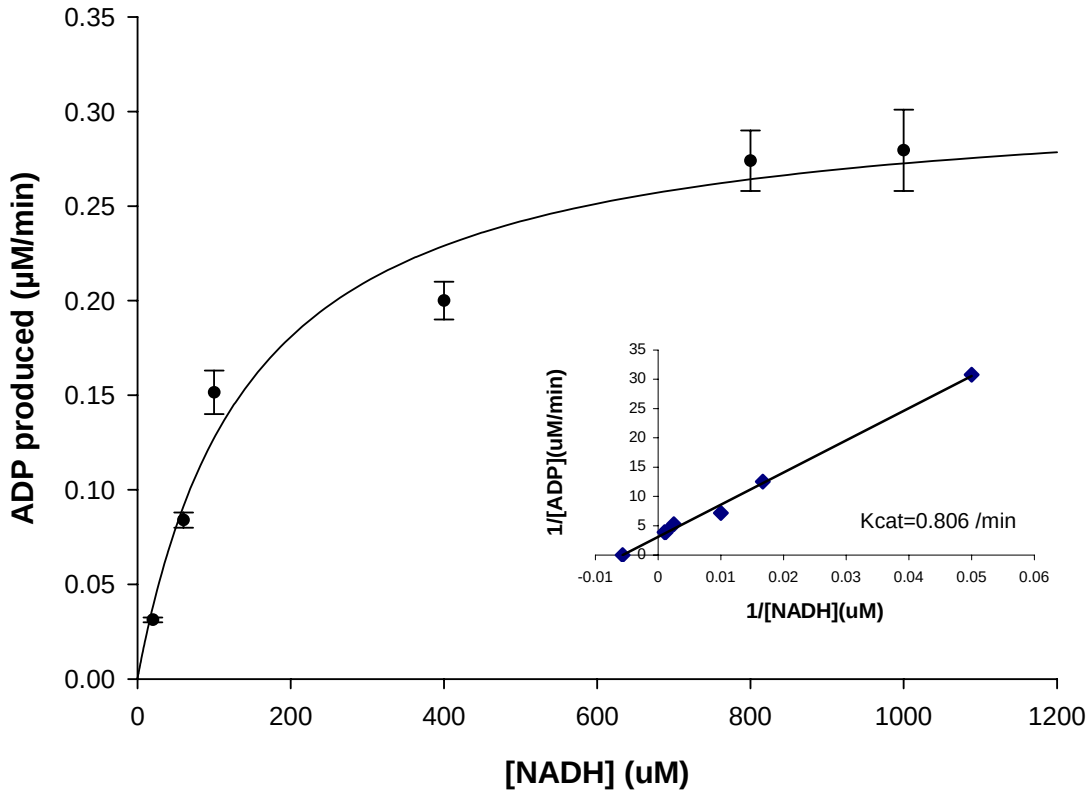


Figure 4.5: Rate of ADP release.

0.4 µM of PfHsp70 and 4 mM of ATP were used to determine rate of ADP release. The curves plotted were used to calculate k_{cat} for ADP relaesed. **k_{cat} was found to be 0.806 min $^{-1}$.**

4.4 CONCLUSION

To date no Basal ATP hydrolysis of PfHsp70 has been shown. Our work has revealed a very low ATP hydroysis that was found to be 4 times and 2 times lower than that of Human Hsc70 and Hsp70 respectively. This low activity may result from the denaturing conditions used during purification, which may result in some proteins not properly folding during native washes and elutions. RCMLA was found to stimulate PfHsp70 by 11 fold whereas Tcj2 and RCMLA stimulated the ATPase activity by 16-fold. Excess Tcj2 in the presence and absence of RCMLA revealed no activity. This may be the result of excess Tcj2 blocking the binding site of ATP/ADP cleft. At the time of experiments Pfjs were not available therefore Tcj2 was used as cochaperone.

The rate of ATP hydrolysis and the rate of ADP release experiments were carried out in order to determine the rate limiting steps during ATPase activity. The calculated k_{cat} (0.003 min^{-1}) of ATP hydrolysis had a lower value than that of ADP release (k_{cat} 0.806 min^{-1}). These results revealed that the rate of ADP release from the ATP/ADP cleft of PfHsp70 was fast compared to the rate of ATP hydrolysis. This may indicate that ATP hydrolysis maybe the rate-limiting step during ATPase activity.

CHAPTER 1

LITERATURE REVIEW

1 GENERAL INTRODUCTION

1.1 CELL STRESS RESPONSE

In all organisms there is an intricate network of stress responses, which ensure that cells and tissues are protected from acute and often toxic changes in the environment (Morimoto *et al*., 1997*)*. Stress has broad connotations with biological systems that change the survival of the cell (Morimoto *et al*., 1997*)*. Exposure to stress results in the inducible expression of diverse stress-responsive gene and proteins, which function to protect the cell and to re-establish homeostasis (Morimoto *et al)*. These include response to extreme temperatures, UV light, oxidants, toxic chemicals, pharmacological active molecules and mutagens. Severe stress often results in a generalized arrest of DNA and RNA synthesis, if prolonged it can lead to withdrawal of the stressed cells from the cell cycle and tissue abnormalities in multicellular organisms due to developmental delays (Morimoto *et al*., 1997*)*. Heat-shock response is a highly ordered pattern of genetically defined responses, which are initiated by stress. Although the exposure of cells and organisms to elevated temperatures represents the prototypical stress. Heat-shock response is induced by conditions as diverse as infection with viral and bacterial agents or exposure to transition heavy metals, amino acid analogues and oxidants (Morimoto *et al*., 1997*)*. The complexity that underlies the transcriptional regulation of heat-shock genes is exemplified by a diverse array of stress conditions, which can be separated into three major categories, including: (i) environmental stress such as heat shock, amino acid analogues, drugs, toxic chemicals and heavy metals; (ii) non-stress conditions, including the cell cycle, growth factors, serum stimulation, development, differentiation and activation by certain oncogenes; and (iii) pathophysiological and disease states, including oxidative stress, fever, inflammation, infection, myocardial stress and ischaemia, neural degenerative diseases and cancer (Figure 1.1)(Morimoto *et al*., 1997*)*. Although the acute response to stress may be critical for the recovery and long-term survival of the affected tissues, the chronic expression of heat shock proteins (Hsps) and molecular

chaperones may also be deleterious for protein biogenesis and cell growth (Morimoto *et al*., 1997*)*.

1.1.1 HEAT SHOCK PROTEINS

Investigations of the cellular response to heat shock and other types of physiological stresses have allowed the identification of families of heat shock proteins (Hsps)(Arrigo *et al*., 1998). Heat shock proteins range in molecular size from 8 to 150 kDa (table 1) (Whitley *et al*., 1998).

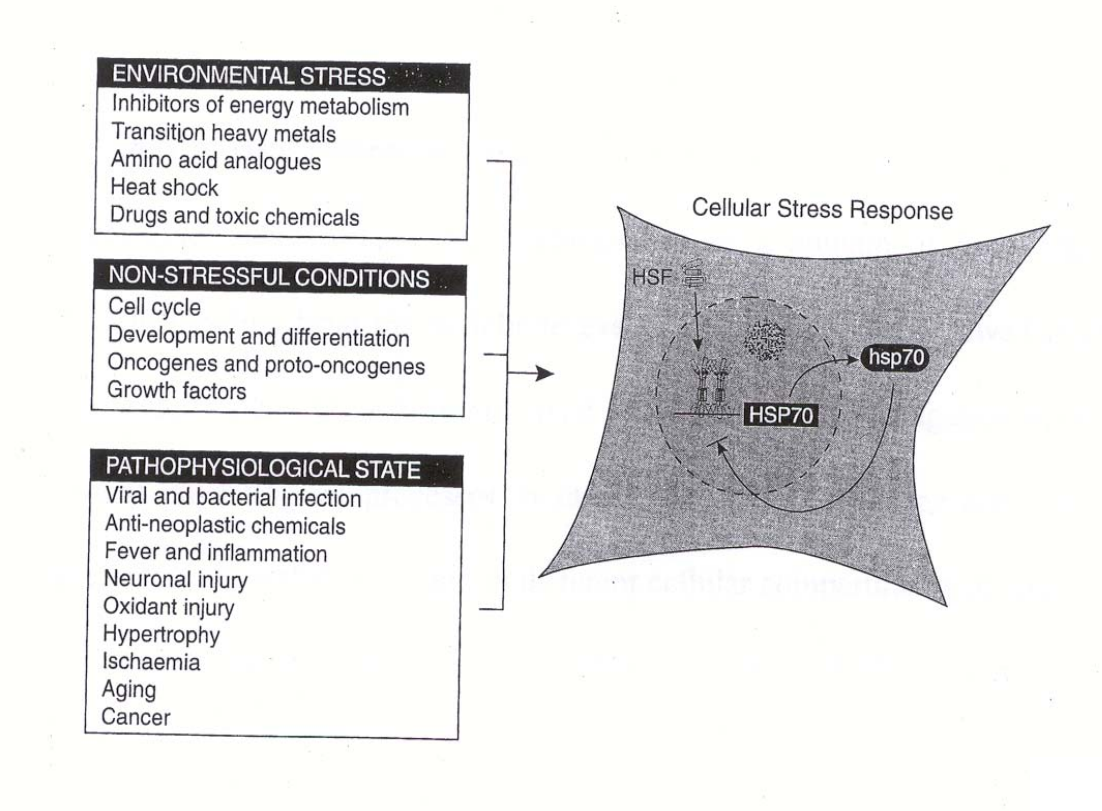


Figure 1.1: Cell stress response. Conditions that induce heat-shock gene expression.The diagram depicts the stress-dependent activity of heat shock transcription factor (HSF), which occurs in response to environmental, non-stressful, and pathophysiology stress resulting in elevated transcription of the heat shock protein 70 (HSP70) gene and expression of heat shock protein 70 (hsp70) (Morimoto *et al.,*1997*)*.

Among these are Hsp60, Hsp70, and Hsp90 families containing proteins that display chaperone function(s) (Arrigo *et al*., 1998). Heat shock proteins are usually named according to their molecular size i.*e:* 70 kDa proteins are referred to as heat shock protein70 (Hsp70) and the gene coding for the protein would be *hsp* 70 (Whitley *et al*., 1998). Stress proteins including the Hsps have been proposed as general marker

of cellular stress and their use for environmental monitoring is often suggested (Ait-Aisse *et al*., 2000). Hsps have generated renewed interest because these proteins may not only be involved in cellular protection against stress, but also in essential physiological processes in unstressed cells (Arrigo *et al*., 1998). Hsps are highly conserved abundant proteins that are found in different cellular compartments including the cytosol, mitochondria, nucleus, nucleolus, endoplasmic reticulum (ER), lysosomes and the plasma membrane (Multhoff *et al*., 1998). Many Hsps are always present in the cell while the expressions of other Hsps are increased by stress. They are rapidly induced through transcription (Whitley *et al*., 1998).

Table 1: Heat shock proteins in eukaryotes (Whitley *et al*., 1998, Smith *et al*., 1998).

	Molecular size (kDa)	Location	Major functions
Hsp40	40	Cytosol/mitorondria/ Nucleus	Stabilization of misfolded proteins,co-chaperone for Hsp70
Hsp60	60	mitochondria	Protein folding (limited substrates in eukaryotic cytoplasm)
Hsp70	70	Cytosol/nucleus	Protein folding, membrane transport of proteins.
Hsp90	90	Cytosol/nucleus	Regulatory interaction with signalling proteins, stabilization of misfolded proteins
Hsp100	100	Cytosol/nucleus	Protein dissaggregation, thermotolerance

Gene transcription is controlled by heat shock transcription factors. Inactive heat shock factors exist as monomers. Once activated, they trimerize into an active form, which is capable of binding to the promoter site of the Hsp gene, which then initiates transcription (Whitley *et al*., 1998). Abnormal levels of Hsps have been found in a number of disorders, including atherosclerosis, congestive heart failure, fever, infection and aging (Whitley *et al*., 1998). Several physical and chemical conditions favour inappropriate folding of proteins and are thus hazardous to cells (Smith *et al*., 1998). It has been recognized that mild temperature elevation can induce heat shock response in cells. This response is characterized by a rapid shutdown of the synthesis of most proteins by Hsp. A similar response occurs after other proteotoxic insults (Smith *et al*., 1998).

1.1.2 MOLECULAR CHAPERONES

Many of the major heat shock proteins i.e. Hsp40, Hsp60, Hsp70, Hsp90 and Hsp100 assist in the folding of newly synthesized proteins, refolding of denatured proteins or promoting their degradation after stress or injury. They are therefore referred to as "molecular chaperones" because of these functions (Figure 1.2) (Whitley *et al*., 1998). Molecular chaperones are critically important because they appear to be necessary in the critical steps of the three-dimensional folding of some newly formed proteins within the cell (Whitley *et al*., 1998). Although the amino acid sequence alone is sufficient to dictate the native conformation of small proteins *in vitro* most polypeptides would fail to fold efficiently in the highly concentrated and complex cellular environment without the assistance of molecular chaperones (Smith *et al*., 1998). Chaperones ensure that newly formed polypeptides proceed correctly through folding and unfolding to eventually achieve a functional shape (Whitley *et al*., 1998).

Many molecular chaperones and their associated co-chaperones are constitutively expressed in all cells (Smith *et al*., 1998). They are found in all component of the cell, whoever the endoplasmic reticulum (ER) contains its own complex chaperone machinery, compared to other component of the cell it provides a highly specialize environment for chaperone activity. The two major chaperones in the ER are the Bip/Grp78 and Grp94/gp96 members of the Hsp70 and Hsp90 families, respectively. Compared to the cytoplasm, the lumen of the ER is a distinct folding environment in which the redox potential is oxidizing and there is a relatively high concentration of calcium (Ca^{2+}) (Smith *et al*., 1998). After chain elongation is complete the incompletely folded nascent chain may undergo sequential rounds of chaperone binding. Only fully folded chains and properly assembled oligomeric complexes exit efficiently from the ER and progress to the Golgi apparatus. Misfolded or unassembled proteins are retained in the ER by continued chaperone interactions but combinations of chaperone interactions vary with different substrates. The ER chaperone machinery has been characterized as a quality control station. Apart from their role in protein folding, several components of the chaperone machinery appear to function as regulatory factors for a variety of signalling proteins. Hsp90 has been

found to interact with multiple regulatory proteins, including the steroid hormone receptors and several transcription factors unrelated to steroid receptors (Smith *et al*., 1998).

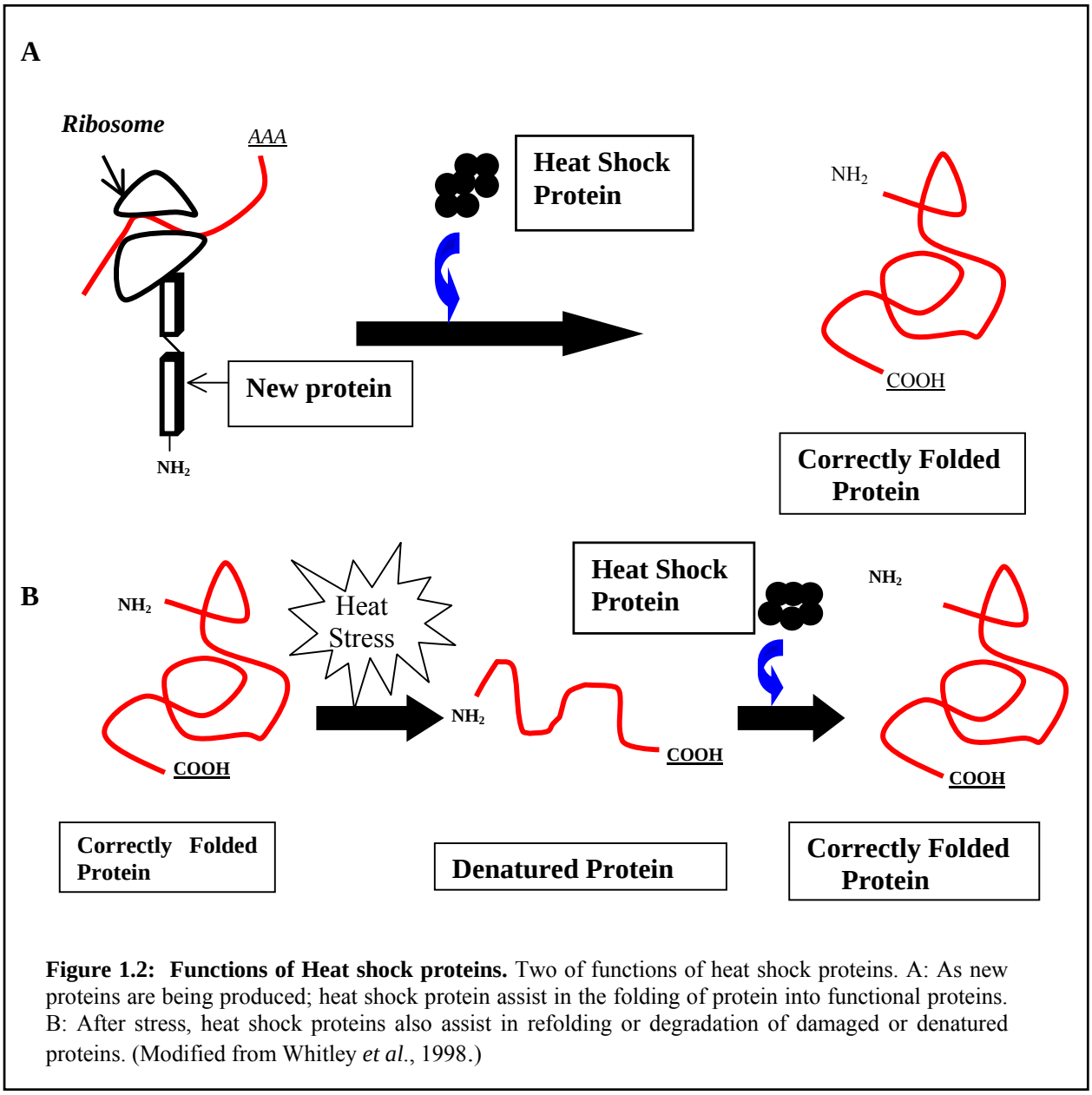


Figure 1.2: Functions of Heat shock proteins. Two of functions of heat shock proteins. A: As new proteins are being produced; heat shock protein assist in the folding of protein into functional proteins. B: After stress, heat shock proteins also assist in refolding or degradation of damaged or denatured proteins. (Modified from Whitley *et al*., 1998.)

Molecular chaperones differ in their ability to stabilize non-native polypeptides and to mediate protein folding defining "holding" and "folding" systems (Luders *et al*., 1998). Mammalian cytosolic and nuclear chaperone Hsc70 can act as both a "holding" and a "folding" system depending on the chaperone cofactors, which associate with it (Luders *et al*., 1998).

1.1.3 HEAT SHOCK PROTEIN 70 (Hsp70)

The diversity of cellular functions of Hsp70 systems poses an intriguing logistic problem (Mayer *et al*., 1998). However strategies have to exist to prevent the sequestration of Hsp70 protein away from their housekeeping targets even under stress. Under-stress conditions the stress inducible heat shock protein (Hsp70) system, is involved in repair of damaged proteins. Heat shock protein (Hsp70) chaperone systems play an essential role in the life cycle of many proteins not only in a hostile environment but also under normal growth conditions. Their activity requires ATP and cooperation with cofactors, which control the Hsp70 ATPase cycle and target the chaperone to specific substrate (Mayer *et al*., 1998). Hsp70 requires the cooperation with members of the family of Hsp40. Hsp70 is built of three domains, an N-terminal ATPase domain of 45 kDa, a central substrate binding domain of 15 kDa and a C-terminal domain of approximately 10 kDa with possibly regulatory functions (figure 1.3) (Mayer *et al*., 1998). The ATPase domain of Hsp70 is nearly identical in structure to the ATPase domain of the constitutively produced heat shock cognate 70 (Hsc70) and they are furthermore both very similar to the protein fold of actin (Mayer *et al*., 1998).

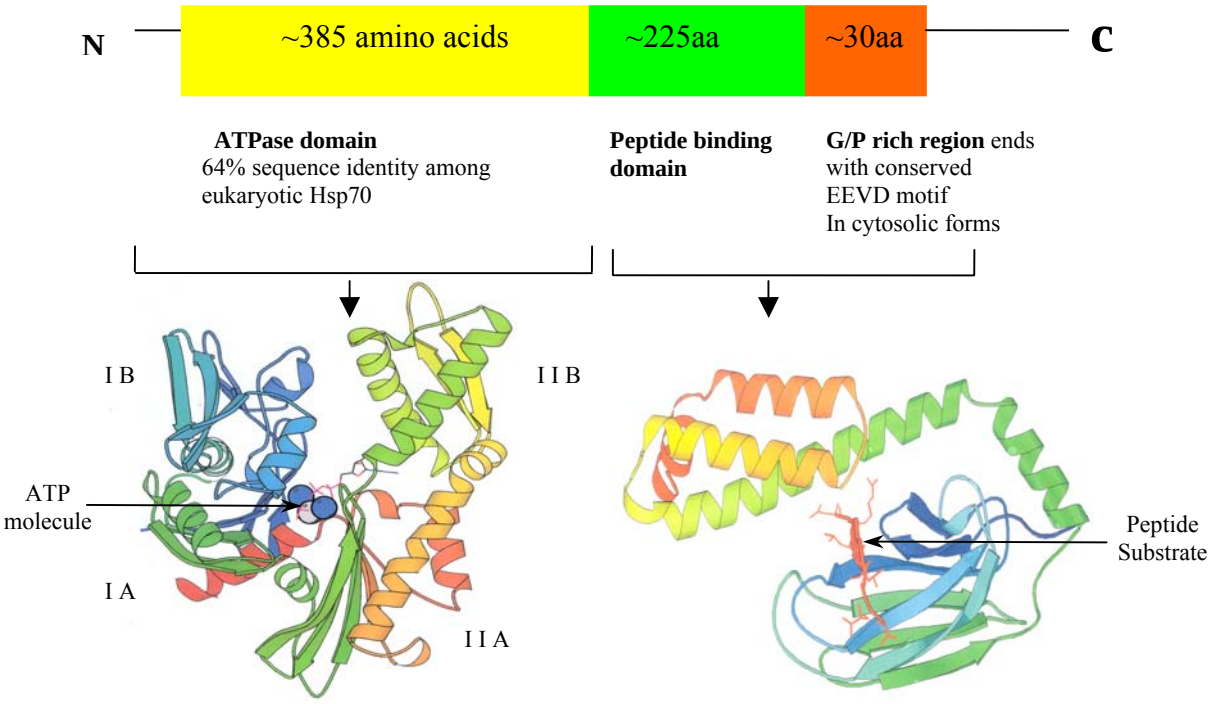


Figure 1.3: Structure of Bovine Hsc70 . The schematic diagram is a linear representation of the domain organization of Hsp70. Approximate numbers of amino acid residues containing a domain are in the boxes. The ribbon diagrams show the ATPase domin and substrate domain. N:N-termius,C:C-terminus. IB,IIB,IA,IIA:subdomains (Modified from Mayer et al., 1998).

1.1.3.1 ATPase DOMAIN

Hsp70 has a common structural feature that consists of two domains of similar fold on either side of a large cleft with an ATP binding site at the bottom of the cleft (Bork *et al*., 1992). Each domain is composed of two subdomains I and II (figure 1.4). Subdomains Ia and IIa have the same basic fold, a central β-sheet surrounded by helices, with identical topology of loop connections (Bork *et al*., 1992). The phosphate tail of the ATP is bound by residues on two β-hairpins, one from each of the subdomains Ia and IIa and the other from nearby segments. This ATP binding mortif is distinctly different from the single phosphate binding loop in such as in adenylate kinase (Bork *et al*., 1992). The ATPase active site are lined with identical or similar residues and the overall domain structure is suggestive of the capacity for interdomain motion perhaps directly coupled to the ATPase activity (Bork *et al*., 1992).

1.1.3.2 SUBSTRATE BINDING DOMAIN

Chaperone activity of Hsp70 in protein folding relies on their ability to repeatedly associate with short stretches of polypeptides in extended conformations (Mayer *et al*., 2000). The reaction is controlled by the binding of ATP to the ATPase domain of Hsp70, which induces conformational changes in the adjacent substrate-binding domain that opens the substrate-binding cavity. This results in high association and dissociation rate constants for substrates (Bork *et al*., 1992). ATP hydrolysis causes the substrate-binding cavity to close, which results in low association and dissociation rate constants for bound substrates. The cochaperone DnaJ (homologue of Hsp40), in synergy with the bound protein substrate in the substrate-binding cavity triggers the cleavage of γ-phosphate the rate-limiting step of the ATPase cycle. The substrate domain consists of a β-subdomain (amino acids 393-501) and C-terminal α-helical subdomain (amino acids 509-607)(figure 1.5). The peptide protein is bound in a cavity formed by two pairs of inner and outer loops protruding upwards from the β-sandwich. This structure suggests that two elements are crucial for peptide binding. There is a hydrophobic pocket that accommodates a single hydrophobic side chain and an arch formed by residues Methionine (404) and Alanine (429) that encloses the

substrate backbone (figure 1.5). Two distinct crystal lattices proposed that residues 536-538 constitute a hinge for a helical lid that is closed in the ADP state but is opened in the ATP state (Mayer *et al*., 2000).

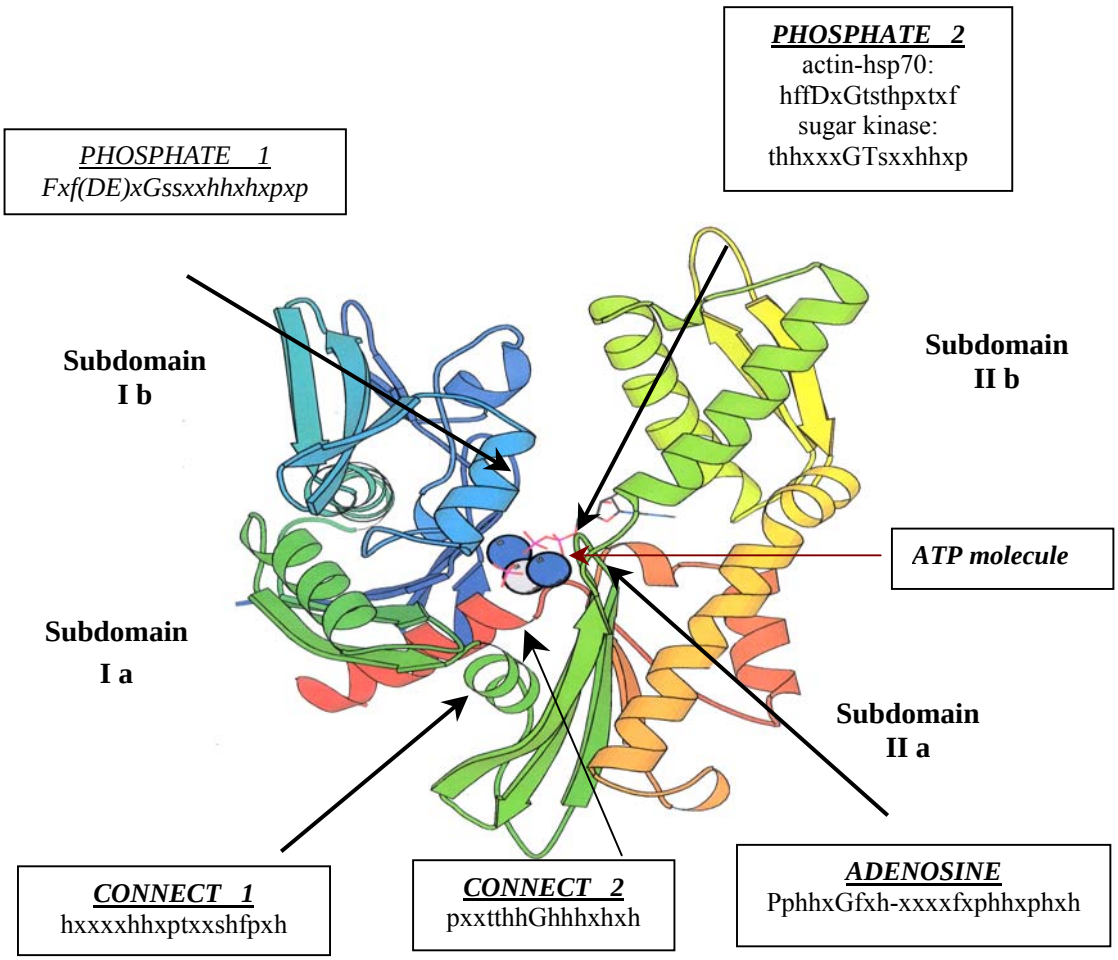


Figure 1.4: Diagramatic ribbon representation of Bovine Hsp70 ATPase domain. Five parts of the sequence pattern, which are characteristic of Hsp70 ATPase domain, are indicated by arrows. The sequence motif of each of the five regions is given in terms of the following amino acid groups: h, purely hydrophobic (VLIFWY); f, partly hydrophobic (VLIFWYMCGATKHR); t, tiny (GSAT); s, small (GSATNDVCP); p, tiny plus polar (GSATNDQEKHR); x, any amino acid.

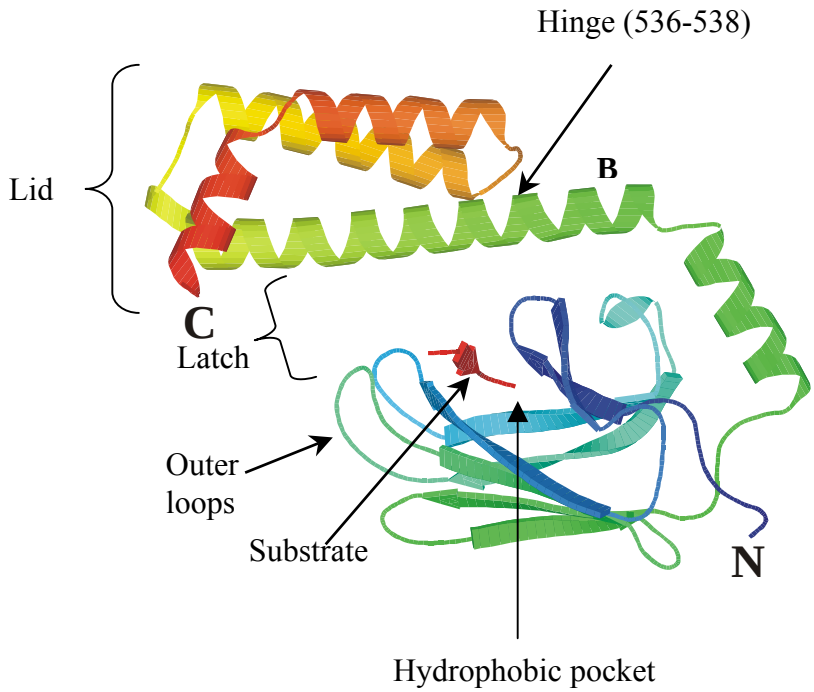


Figure 1.5: Diagramatic representation of Bovine Hsp70 substrate binding domain. Hsp70 substrate binding domain has amino acids that interact with the substrate peptide. The latch is formed by a salt bridge and hydrogen bonds between amino acids of the distal part of helix B and the outer loops.

1.1.3.3 C-TERMINAL (EEVD MOTIF)

In the folding of proteins such as steroid hormone receptors, Hsp70 and Hsp90 interact with cofactors such as Hop (Hsc70-Hsp90 organizing protein), which contain tetratricopeptide repeat (TPR) domains (Brinker*et al*., 2002). TPR domains contain degenerate 34 amino acid motifs that are found 1-16 times per domain. (Blatch and Lässle, 1999). TPR motif contains two antiparallel α-helices such that tandem arrays of TPR motifs generate a right-handed helical structure with an amphipathic channel that might accommodate the complementary region of a target protein (Blatch and Lässle, 1999). Most TPR motifs are important to the functioning of chaperone, cell cycle and protein transport complexes (Blatch and Lässle, 1999). The TPR domain interact with the C-terminal domain of Hsp70 (Brinker*e tal*.,2002). Deletion

mutagenesis of the highly conserved EEVD-COOH motif of Hsp70 in eukaryotes has shown it to have great importance in the binding to the N-terminal TPR domain (TPR I) of HOP (Brinker.*et.al*., 2002). The TPR I domain of HOP recognises the C-terminal seven amino acids of Hsp70 (*PTI*EEVD). Deletion or mutation of the EEVD has an affect on the ATPase activity and also on the interaction with substrate, it also affects the interaction with Hsp40 (HDJ-1) (Freeman *et al*., 1995). As a result the EEVD motif is involved in the intramolecular regulation of Hsp70 function and intermolecular interaction with HDJ-1 (Freeman *et al*., 1995).

1.1.4 HEAT SHOCK PROTEIN 40 (Hsp40)

DnaJ was first identified in *Escherichia coli (E.coli)* as a molecule, which is encoded in the operon as the major heat shock protein, DnaK (Watanabe 1997). DnaJ in *E.coli* can be subdivided into four domains on the basis of its primary amino acid sequence. The N-terminus consists of the J domain, a 70 amino acid α-helical region. The J-domain defines this family of proteins and some members contain only this domain structure. A primary binding determinant to Hsp70 is wildly believed to be a universally conserved tripeptide, His-Pro-Asp (HPD), which is located in the loop between helices II and III of the J-domain (figure 1.6). Point mutations in this tripeptide abolish binding of DnaJ to DnaK (Hsp70). Work done by Suh (Suh *et al*., 1998) provided evidence that the lower cleft of the N-terminal ATPase domain is a binding pocket for the J-domain. Cheetham and Caplan (1998) have proposed a nomenclature to verify the differences between the DnaJ families based upon their domain conservation. Type I DnaJ proteins have full domain conservation: a J domain of about 70 amino acids at the N-terminal followed by a glycine/phenylalnine rich (over 40% glycine and 15% phenylalanine) region (G/F), which is a flexible linker, next to the G/F region is a cysteine rich region, which is organized into tertiary structure with the help of two Zn^{2+} atoms. Type II has a J domain at the N-terminal and a G/F region that is longer (70-100 amino acids) with a more variable glycine content than Type I, it contain no cysteine region. Type III proteins have only a J domain found anywhere in the protein (Cheethem and Caplan 1998) (figure 1.6).

with *E.coli* DnaJ; Type II has a J domain and a glycine/phenylalnine (G/F) domain normally found at the N-terminus; and Type III proteins have only a J domain found anywhere in the protein (Cheethem and Caplan 1998) (figure 1.6). The C-terminal is less conserved among different J proteins (Cheethem and Caplan 1998).

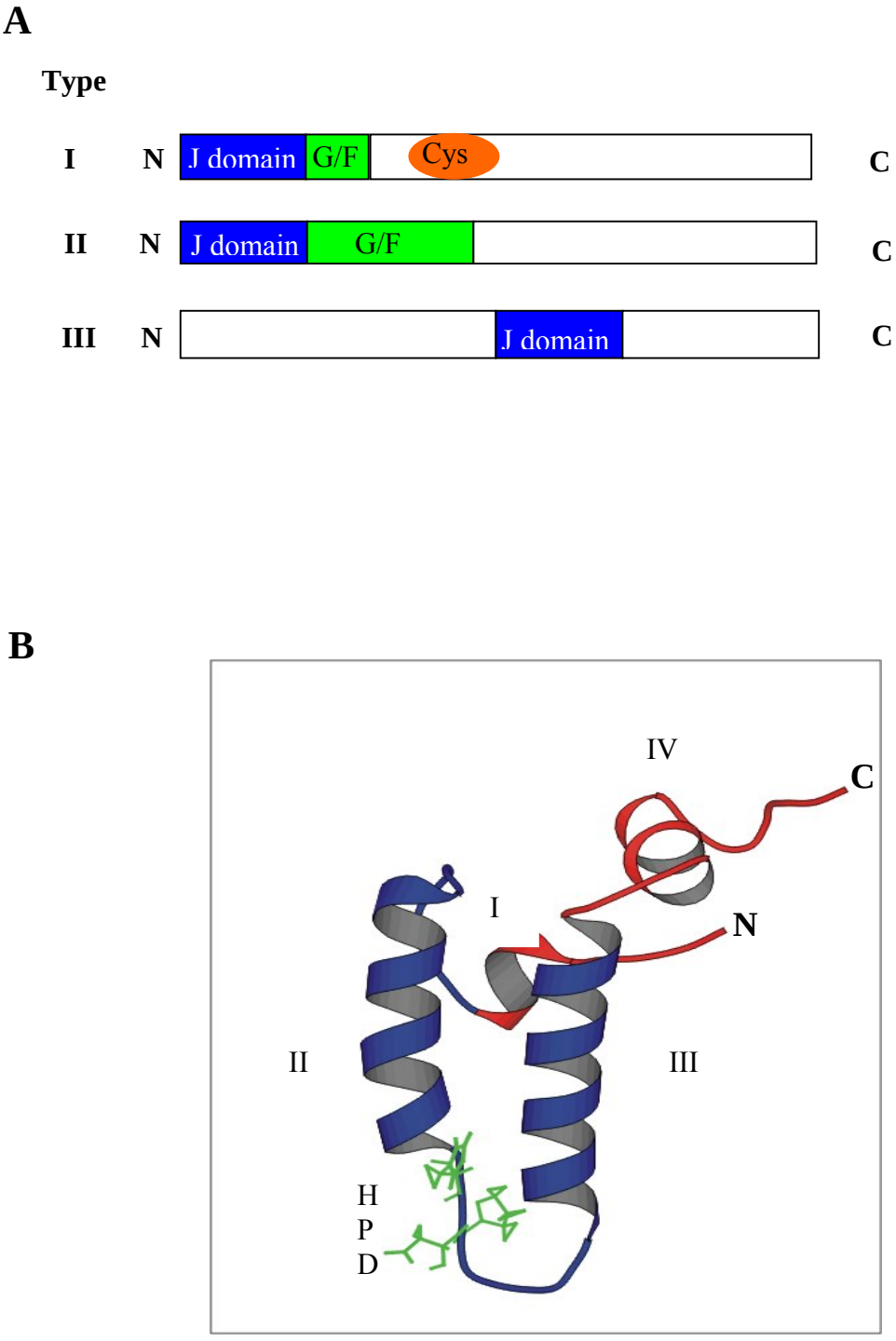


Figure 1.6: Structure of DnaJ.

Linear representation of DnaJ family sub-types (modified from Cheethem and Chaplan 1998). (B) Ribbon diagram of the J-domain of DnaJ was generated using WHATIF soft ware (Chinea *et al*., 1996) and visualized using Molscript soft ware (Peitsch, 1995). N: N-terminus, C: C-terminus, HPD: His-Pro-Asp Cys- cysteine (CysxxCysxGxG) and G/F: glycine/phenylalnine

1.1.5 Hsp70 CHAPERONE MECHANISM IN PROKARYOTES.

The Hsp70 homologues in prokaryotes (DnaK in *E.coli*) and mitochondria require cooperation with a nucleotide exchange factor, GrpE (Mayer and Bukau 1998). DnaK is involved in activity control and rapid turnover of unstable regulatory proteins (Mayer and Bukau 1998). DnaK interacts with GrpE to enhance ADP release, thus allowing the unfolded substrate to dissociate from the Hsp70/DnaJ/GrpE complex, which is also known as the hsp70 chaperone machinary (Morimoto *et al*., 1997*)*. The activity of DnaK protein rely on cycles of substrate binding and release which are driven by conformational changes in DnaK that require the energy of ATP and that are collected by cofactors (Mayer and Bukau 1998). The cycle starts with the transient association of DnaJ with a substrate polypeptide.

DnaJ substrate complex binds to DnaK-ATP allowing the substrate to be transferred into the DnaK binding pocket and the subsequent stimulation of the ATP hydrolysis of DnaK via the J-domain. ATPase regulates the affinity of Hsp70 for substrates (Laufen *et al*., 1999). Hsp70 exhibits low affinity and fast exchange rates for substrates in the ATP state, and high affinity and low exchange rates in the ADP state. Hydrolysis of ATP thus locks substrates into the substrate-binding cavity of Hsp70 (Laufen *et al*., 1999). The substrate becomes stably bound to DnaK-ADP and DnaJ leaves the ternary complex. The assumed role for DnaJ cochaperone is to target Hsp70 partner proteins to their substrates by catalysing ATP hydrolysis by Hsp70 (Laufen *et al*., 1999). The presence of DnaJ promotes the DnaK for efficient DnaK-substrate complex formation (Wawrzynow *et al*., 1995). ATP hydrolysis is absolutely required for such DnaJ-dependent activation of DnaK for binding to both native and denatured protein substrates. In the presence of DnaJ and ATP, DnaK possesses the affinity to different substrates (Wawrzynow *et al*., 1995). The binding of GrpE releases ADP and thereby allows the rapid rebinding of ATP, thus converting DnaK in to the low affinity form for substrates. The substrate is released and may either fold or rebind to DnaJ and re-enter a new cycle of binding and release to DnaK (Figure 1.7) (Mayer and Bukau 1998).

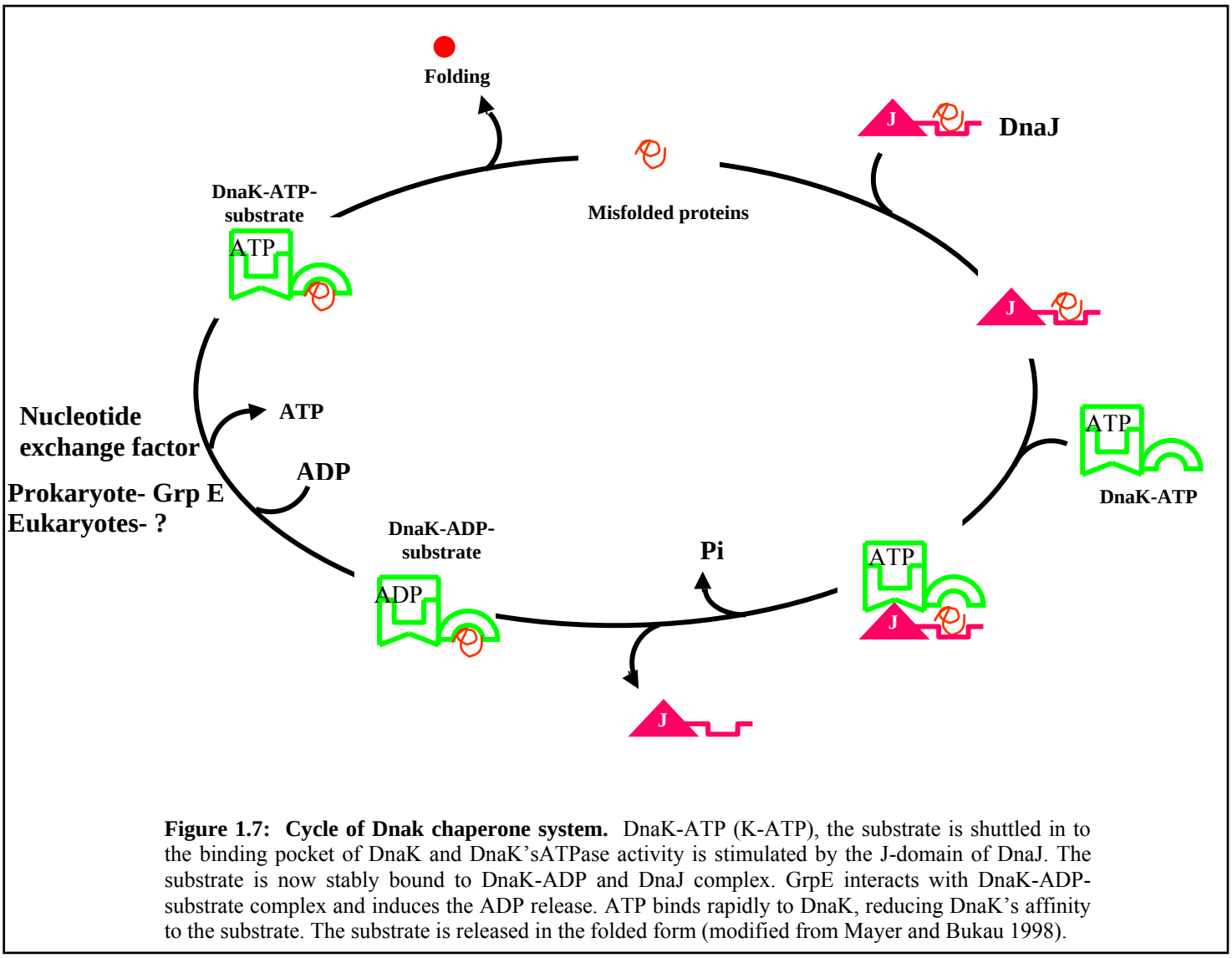


Figure 1.7: Cycle of Dnak chaperone system. DnaK-ATP (K-ATP), the substrate is shuttled in to the binding pocket of DnaK and DnaK'sATPase activity is stimulated by the J-domain of DnaJ. The substrate is now stably bound to DnaK-ADP and DnaJ complex. GrpE interacts with DnaK-ADP-substrate complex and induces the ADP release. ATP binds rapidly to DnaK, reducing DnaK's affinity to the substrate. The substrate is released in the folded form (modified from Mayer and Bukau 1998).

1.1.6 Hsp70 CHAPERONE MECHANISM IN EUKARYOTES.

In eukaryotes several cofactors have been identified which associate and cooperate with Hsc70 (Luders *et al*., 1998). Association with cofactors is essential for the regulation of the ATPase cycle of Hsc70. Altering the affinity of the chaperone for nonnative polypeptide substrates. Hsc70 in the ATP-bound conformation rapidly binds and releases a polypeptide substrate; stable substrate binding depends on the transfer of the chaperone to the ADP-bound state. ATP hydrolysis by Hsc70 is stimulated by cofactors of the Hsp40 protein family, such as Hsp40 (Hdj-1, Hdj-2, and auxilin). Hsp40 is thus required for efficient substrate recognition by Hsc70 ADP-bound state of Hsc70. The generated through an interaction with Hsp40, is affected by the Hsc70 cofactors Hip (Hsp70-interacting protein) and BAG-1 in opposite ways.

Hip slows down the release of ADP from Hsc70 and may therefore stabilize an Hsc70/substrate complex. BAG-1 accelerates ADP release from the chaperone protein, thus strongly stimulating Hsc70's ATPase activity in conjunction with Hsp40. (Hip) and BAG-1 both bind to the ATPase domain of Hsc70 in a mutually exclusive manner. Hip and BAG-1 represent competing Hsc70 cofactors that conversely affect the ADP-bound state of the chaperone protein (Luders *et al*., 1998).

Hsp70 assists in the folding of at least some newly synthesized polypeptides and translocation of proteins across the membranes, for example the ER acting on both sides of the membrane and degradation of proteins. The Hsp70s are also involved in intracellular vesicle trafficking and signal translation. The ability of Hsp70 to bind promiscuously to folding proteins and selectively to native proteins relies on the ATP-regulated association of Hsp70 with short hydrophobic segments of substrate polypeptides. Such association allows Hsp70 to prevent further folding and aggregation of unfolded proteins, by shielding exposed hydrophobic segments of polypeptides to assist their refolding probably by decreasing the concentration of aggregation prone folding intermediates and to modulate the biological activity of folded substrates (Mayer *et al*., 1998).

1.2 MALARIA *PLASMODIUM FALCIPARUM*

1.2.1 LIFE CYCLE OF PLASMODIUM FALCIPARUM.

There are four different types of malaria parasites; these are *Plasmodium falciparum, Plasmodium vivax, Plasmodium malaria,* and *Plasmodium ovale*. Of the four species of *Plasmodium* that cause malaria in humans, *Plasmodium falciparum* is the most dangerous as the pathology it induces often leads to death (Hyde 2002). It can be fatal within few hours of the first symptom. The life cycle of *P. falciparum* is complex; Figure 1.9 shows a diagrammatic representation of the cycle. (a) In humans *P. falciparum* infection begins when an infected *Anopheles* Mosquito takes a blood meal thereby injecting infected sporozoites into the peripheral circulation; (b) sporozoites migrate and invade the hepatocytes within the liver and undergo asexual multiplication, producing merozoites froms of the parasite; (c) infected hepatocytes

rupture releasing merozoites into the peripheral circulation; (d) the merozoites invade red blood cells (rbcs); and (e) complete another round of multiplication within 48-72 hours. (This additional multiplication results in the destruction of rbc haemoglobin in the process); (f) the released merozoites invade more rbcs therefore carrying on the cycle; the release of merozoites is thought to be responsible for the periodic fevers associated with malaria; (g) some merozoites do not divide but form male (microgametocytes) and female (macrogametocytes) sexual forms; (h) these sexual forms are taken from the blood by a feeding *Anopheles* Mosquito; and (i) fertilize in the mosquito midgut to from zygotes, which differentiate into motile forms (ookinetes), which migrate through the mosquito gut and divide into oocytes on the external gut wall to form sporozoites; (j) sporozoites are released into the mosquito haemocoele and move to the salivary gland, where they await injection into another human host, thus completing the cycle (http://www-emm.cbcu.ac.uk).

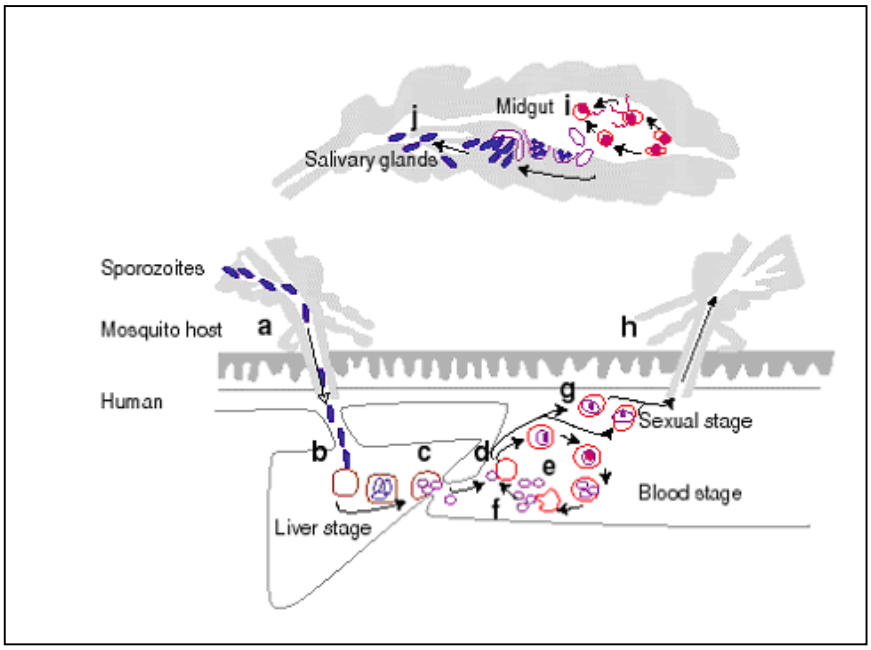


Figure 1.8: Life Cycle of *Plasmodium falciparum*. Cycle shows different stages and forms undertaken by *falciparum* in mosquito and human hosts**.** (a): infection in to human host, (b):invasion of parasites in hepatocytes, (c): rupture of infected hepatocytes, (d): invasion of red blood cells, (e): multiplication cycle, (f): invasion of more rbc, (g): formation of sexual forms (microgametocytes and macrogametocytes), (h): transfer of sexual forms into mosquito, (i): fertilization in mosquito, (j): release of sporozoites into mosquito haemocoele (http://www-emm.cbcu.ac.uk).

1.3 HEAT SHOCK PROTEINS IN *PLASMODIUM FALCIPARUM*.

Like many prokaryotes and eukaryotes the malaria parasite also synthesizes several stress proteins (Sharma, 1992). Techniques like recombinant DNA technology have been done on the malarial Hsp. Five Hsp-encoding genes from *Plasmodium falciparum* have been identified which are located on different chromosomes. There are genes for three Hsp70 and one Hsp90 of the *P. falciparum* have been cloned. These genes are classified as PfHsp70-I, PfHsp70-II, and PfHsp90. The biological roles of these proteins in malaria are not fully understood but it is possible that they provide protection to the parasite from various stresses encountered in the host (Sharma, 1992). In this process Hsps probably bind to the toxic molecules as well as damaged proteins so as to remove them out of the parasite. Their involvement in the stage–specific parasite transformation to increase the infectivity and virulence as observed in other parasites, remains to be determined. Malaria Hsps are antigenic in

humans. This antigenicity could be attributed to the non-homologous sequences in the C-terminal region (Sharma, 1992). The upstream sequence of the Hsp-encoding gene contains several conserved regions. These regions are known as heat shock elements (HSE), where the transcriptional activator, heat shock factor (HSF), binds to initiate transcription. At high temperatures however this factor (HSF) binds more efficiently to HSE, thus enhancing the transcription of the Hsp-encoding genes (Sharma, 1992).

1.4 *PLASMODIUM FALCIPARUM* Hsp70 (PfHsp 70)

P. falciparum Hsp70 (PfHsp70) has an apparent molecular mass of 70 kDa. PfHsp70 is synthesized by all asexual blood stages of the parasite, and reported to be a soluble cytoplasmic protein (Sharma, 1992). PfHs70 are barely detected in the salivary gland sporozoites (Kumar and Zheng 1992). They are expressed at elevated levels in the parasites undergoing development in liver cells (exoerythrocytic stages). Temperature shift studies in blood-stage parasites of the *P. falciparum* have also shown that the Hsp70 like protein is heat inducible. PfHsp70 is located in the nuclear and cytoplasmic compartments of the parasite (Kumar and Zheng, 1992).

1.4.1 EFFECT OF HEAT ON PfHsp70

The effect of various body temperatures encountered during malarial fever on the synthesis of *P.falciparum* heat shock protein PfHsp70 and parasite growth rates among 5 different isolates were carried out (Biswas and Sharma, 1994). The results showed that after the exposure of the parasite to 39°C for 30 minutes, the amount of PfHsp70 in all 5 isolates increased markedly and significantly whereas parasite growth rates and the amount of total blood stage antigens remained almost unaffected. This indicated that the PfHsp70-encoding gene responded to heat shock by producing higher amounts of PfHsp70 proteins presumably to protect the parasite from being killed during malaria fever (Biswas and Sharma, 1994). In various experiments *P. falciparum* was subjected to heat shock for varying times and temperatures in order to test for their viability, growth, and expression (Joshi *et al*., 1992). Results showed that the majority of parasites remained viable after heat shock but their growth was affected. Those growing at 37°C grew faster compared to those exposed to 35°C, 39°C or 41°C for certain periods of time before returning them to 37°C for 48 hours.

At 41°C the maximum number of parasites were found dead after heat-shock, the death rate increasing if they are exposed for 30 minutes or longer (Joshi *et al*., 1992). PfHsp70 gene are expressed and enhanced after heat shock, this results in the malaria parasites being able to survive *in vivo* during fever, this is probably due to their overexpression (Joshi *et al*., 1992).

1.4.2 IMMUNOLOGY OF PfHsp70

Frequently antibodies against heat shock proteins are generated as a result of parasite infection and the best characterized is the *Plasmodium* chaperone member of the Hsp70 family (Tsuji *et al*., 1994). Different *P. falciparum* Hsp70 genes have been identified. Three monoclonal antibodies generated by immunization of mice with *P. berghei* infected red blood cells were found to react with the Hsp70 (Tsuji *et al*., 1994). These monoclonal antibodies react not only with sporozoites of *P. berghei* but also with sporozoites of several other rodent and human *Plasmodium* species (Tsuji *et al*., 1994). Specific antibodies against PfHsp70 were detected in the sera of individuals exposed to the parasite (Kumar *et al*., 1990). *P. falciparum* from different geographical locations showed conserved genes for Hsps, thus they are likely to be immune targets in various epidemic areas (Kumar *et al*., 1990). Lymphocytes from two tested immune donors responded in proliferation assays to purified PfHsp70 and recombinant protein (PfHsp70). Similar response was seen in lymphocytes from non-immune individuals and raised questions pertaining to a generalized responsiveness of lymphocytes to some common determinants present in heat shock-related proteins in various pathogens (Kumar *et al*., 1990).

A gene was cloned that had encoded a novel protein with sequence domains, some similar to the Gly – Gly – Met – Pro (GGMP) repeat region found near the carboxyl terminus of the Hsp70 of *P. falciparum*. The other exhibited highly significant resemblance to analogous acidic and basic domains found in numerous eukaryotic transcription factors (Uparanukraw *et al*., 1993). Early Hsps of a wide variety of organisms have been found to be targets of cellular and humoral immune response (Kumar and Zheng, 1998). Hsp70 of the malaria parasite *P. falciparum* have been shown to be targets of the natural immune response. Antibodies recognize specific epitopes in PfHsp70 and T –cells which are targets of antibody – dependent cell-mediated cytotoxicity. It was found that the GGMP repeat region in the PfHsp70

immunogen was a dominant epitope recognized by large proportion of mice. Further evidence to show a dominant antibody response against the GGMP epitope came from the studies in which the peptide was conjugated to the carrier molecule Bovine serum albumin (BSA).

1.4.3 STRUCTURE OF PfHsp70

Cloned PfHsp70 family from *P.falciparum* of 75 kDa and 72 kDa were shown to share sequence similarities with eukaryote Hsp70 (Kumar and Zheng, 1992). Like eukaryotic Hsp70 they contain three domains, an N-terminal ATPase domain of 45 kDa, a central substrate binding domain of 15 kDa and a C-terminal domain of approximately 10 kDa. At the C-terminal there is a highly conserved GGMP motif that was shown to cause an immune response in mice (Kumar and Zhenga, 1998). Pfhsp70 contains a conserved EEVD motif also found in Hsp70 of eukaryote, which binds to TPR motif of HOP.

1.5. HEAT SHOCK PROTEIN DnaJ HOMOLOGUES FROM *PLASMODIUM Falciparum*(Pfj 1).

The complete sequence of a DnaJ homologue, Pfj1 from *P.falciparum* was determined by Watanabe (Watanabe,1997). Comparison of Pfj1, DnaJ and Mdj1 (mitochondrial DnaJ homologue of *Saccharomyces cerevisiae*) revealed five characteristic regions. Four repeats of Cys-x-x-Cys-x-Gly-x-Gly (204-254 aa) were found which resembled a zinc-finger motif. It was found that this region actually binds two zinc ions and functions in substrate-binding. Down stream of the repeats is a region with 140 amino acids (270-403 aa), which also contain conserved amino acids. The determined sequence contained 1881 nucleotides encoding 627 amino acids. Pfj1 contained an additional 200 amino acid (424-627 amino acid) with a stretch rich in lysine and proline. Pfj1 belongs to the classical DnaJ group which functions in prokaryotes or mitochondria. According to the classification in Cheetham (Cheetham *et al* 1998) Pfj1 belongs to Type I. Type I have full domain conservation with DnaJ. Pfj1 may play important roles in the adaptation to homeothermal vertebrate hosts (Watanabe, 1997).

1.6 HYPOTHESIS, AIMS AND OBJECTIVES.

No specific work has been carried out on PfHsp70; therefore it's ability to act as achaperone has not been detected.

1.6.1 HYPOTHESIS

PfHsp70 is involved in cytoprotection of the malaria parasite through its action as a molecular chaperone. It assists in the folding and refolding of newly synthesized and denatured proteins. During heat stress conditions its chaperone activity is increased resulting in the survival of the malarial parasite.

1.6.2 AIM

The aim of this study was to investigate the chaperone activity of PfHsp70. The protein was expressed and purified using a heterologous expression system.

1.6.3 OBJECTIVES OF THIS STUDY

There are several objectives investigated in this work. Firstly, the similarities of PfHsp70 was compared to those of eukaryotes and prokaryotes using bioinformatic tools and a three dimensional structure was generated using homology modelling. Secondly, overproduce and purification recombinant PfHsp70 was achieved using Ni-chelating affinity. Thirdly, the basal ATP hydrolysisand stimulated levels of ATP Hydrolysis in the presence of substrate and cochaperone were determined. Fourthly, the molecular mass of PfHsp70 was determined using gel filtration chromatography. Fifthly, Binding studies were were carried out using reduced and carboxymethylated lactalbumin (RCMLA) as substrate.

CHAPTER 2

BIOINFORMATIC ANALYSIS OF PfHsp70 AND Pfjs

2.1 INTRODUCTION

2.1.1 BIOINFORMATICS

Bioinformatics is the computer-assisted data management discipline that enables the collection anaysis and representation of biological information in order to understand life's processes (Combet *et al*., 2002). This new field of science fuses biology, medicine, mathematics, statistics and computer science (http://www.math.chalmers.se/Stat/Bioinfo/General/intro.html). Bioinformatics has played a major role in protein engineering and enables homology modelling of predicted molecules. Homology modelling gives a starting point for biologists involved in protein structure-function relationship studies when the experimental structure is not available (Combet *et al.,* 2002). Prediction of functional residues, and secondary structures relay on the analysis of multiple protein sequence alignment (Cuff *et al.,* 2000). Greater reliability in the function and structure prediction may be obtained by increasing the number of sequences in the multiple sequence alignment (Cuff *et al.,* 2000). Homology modelling restraints derived from a structural three dimensional template are used to fold the query sequence in the distance geometry step. Templates may be obtained from homologous proteins that are identified by a sequence similarity search (Combet *et al.,* 2002).

Homology modelling tools have been developed such as the Swiss-model web server, which contains protein sequence databases such as SWISS- PROT and these may often contain homologues to the sequences of interest (Cuff *et al.,* 2000; Combet *et al.,* 2002). A software known as JavaShade is a multiple sequence alignment box and shade tool that was designed to generate publication quality Postscript output (Southern and Lewis, 1998). Boxing and shading of residues within the multiple sequence alignment is one of the most informative ways of representation (Southern and Lewis 1998). The degree of sequence conservation across the alignment is highlighted in order to identify specific features within the alignment (Southern and Lewis, 1998). There are several software tools that are able to box and shade multiple sequence alignments (Southern and Lewis 1998).

2.1.2 OBJECTIVE

Very limited work has been carried out on identifying important residues and determining the structure of PfHsp70. The broad objective of this study was to apply bioinformatic tools, such as primary sequence alignments and homology modelling to identify structural features potentially of functional importance. Functional motifs and domains were identified by searching for conserved blocks within multiple sequence alignment of Hsp70s from eukaryotes and prokaryotes. The predicted three-dimensional structure was generated by WHATIF computer program (Chinea *et.al.,* 1996) and visualised using MOLSCRIPT (Kraulis, 1991), allowing identification of domains and positions of important residues.

2.2 EXPERIMENTAL MATERIALS AND MEHTODOLOGY

2.2.1 BIOINFORMATIC ANALYSIS

Protein sequences were obtained from the Gene bank browser server (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) and aligned using the BCM Search Launcher: multiple Sequence Alignments (http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html). WHATIF (Chinea e*t al.,* 1996) was used to model the three dimensional structure of PfHsp70 using structure of human Hsc 70 (PDB code 3HSC). The three dimensional structure was visualized using MOLSCRIPT soft ware (Kraulis, 1991).

2.3 RESULTS AND DISCUSSION

2.3.1 MULTIPLE SEQUENCE ALIGNMENT OF PfHsp70

2.3.1.1 N-TERMINUS (ATPase DOMAIN) OF PfHsp70s

An amino acid alignment of PfHsp70 and its homologues was produced to determine important conserved residues of the N-terminal ATPase domain and the C-terminal domain (Figures 2.1 and 2.2).

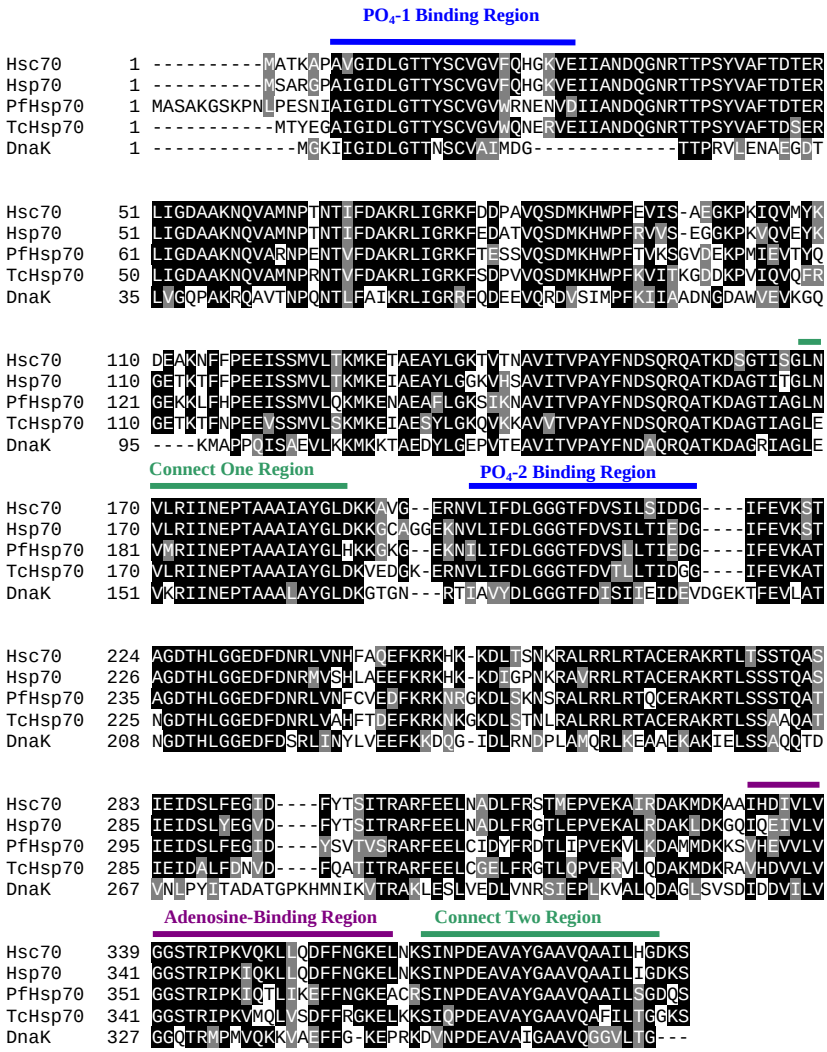


Figure 2.1:Multiple sequence alignment of the ATPase Domains of Hsp70 homologues.
PfHsp70: *Plasmodium falciparum* heat shock protein 70 (GenBank: P11144); Human Hsc70: Human Heat shock cognate protein 70 (GenBank: P19120); Hsp70 Homo sapiens heat shock protein 70 (GenBank NP068814), TcHsp70: *Trypanosoma cruzi* heat shock protein 70 (GenBank: P05456) and DnaK: *Escherichia coli* heat protein 70 (GenBank: BAA01595). Blue (Phosphate 1 and 2 binding region), Green (Connect 1 and 2 binding region), Purple (Adenosine phosphate –binding region). The black regions show identical and the grey shows similar amino acids. Phosphate 1 and 2 regions: binds to the phosphate tail of ATP Connect 1 and 2 regions: make a helix-helix contact that appears to have the prosperities of an inter-domain hinge, and Adenosine regions: binds to the adenosine molecule of ATP.

A BLAST search was performed in order to identify PfHsp70 homologues; sequences from eukaryotes, prokaryotes and parasites were aligned in order to find identical and similar residues amongst various organisms. Hsc 70 was used because it served as a template for homology modelling of PfHsp70 as the alignment showed a 70% identity between the two proteins therefore allowing Hsc70 to be used as template. The alignment of PfHsp70 homologues revealed a highly conserved N-terminal ATPase domain (Regions in black represent identical while the grey represents similar residues). Several regions are important for function these are shown in Figure 2.1. The Phosphate 1 and 2 regions, which, binds to the phosphate tail of ATP. The Connect 1 and 2 regions make a helix-helix contact that appears to have the properties of an inter-domain hinge. The Adenosine regions, bind to the adenosine molecule of ATP. These regions show highly conserved residues.

2.3.1.2 SUBSTRATE BINDING DOMAIN AND C-TERMINAL OF PfHsp70

The peptide substrate binding, and lid and hinge region show highly conserved residues among the Hsp70. When ATP binds and is hydrolysed to ADP, the lid region tends to close and open in its ATP form. A conserved Gly – Gly – Met – Pro (GGMP) repeat motif is found at the C-terminus. This motif is characteristically found in Hsp70s of parasitic origins and, it is found in PfHsp70 and TcHsp70 (Figure 2.2). It is believed to stimulate an immune response during infection (Kumar and Zheng, 1998). An EEVD motif represented with a blue box is found in all eukaryotic Hsp70s, This region together with a region in Hsp90, is known to interact with Hop (Hsc70-Hsp90 organizing protein) by binding to the tetratricopeptide repeat (TPR) domain of Hop during the folding of proteins such as steroid hormone receptors (Bricker, *et al.,* 2002).

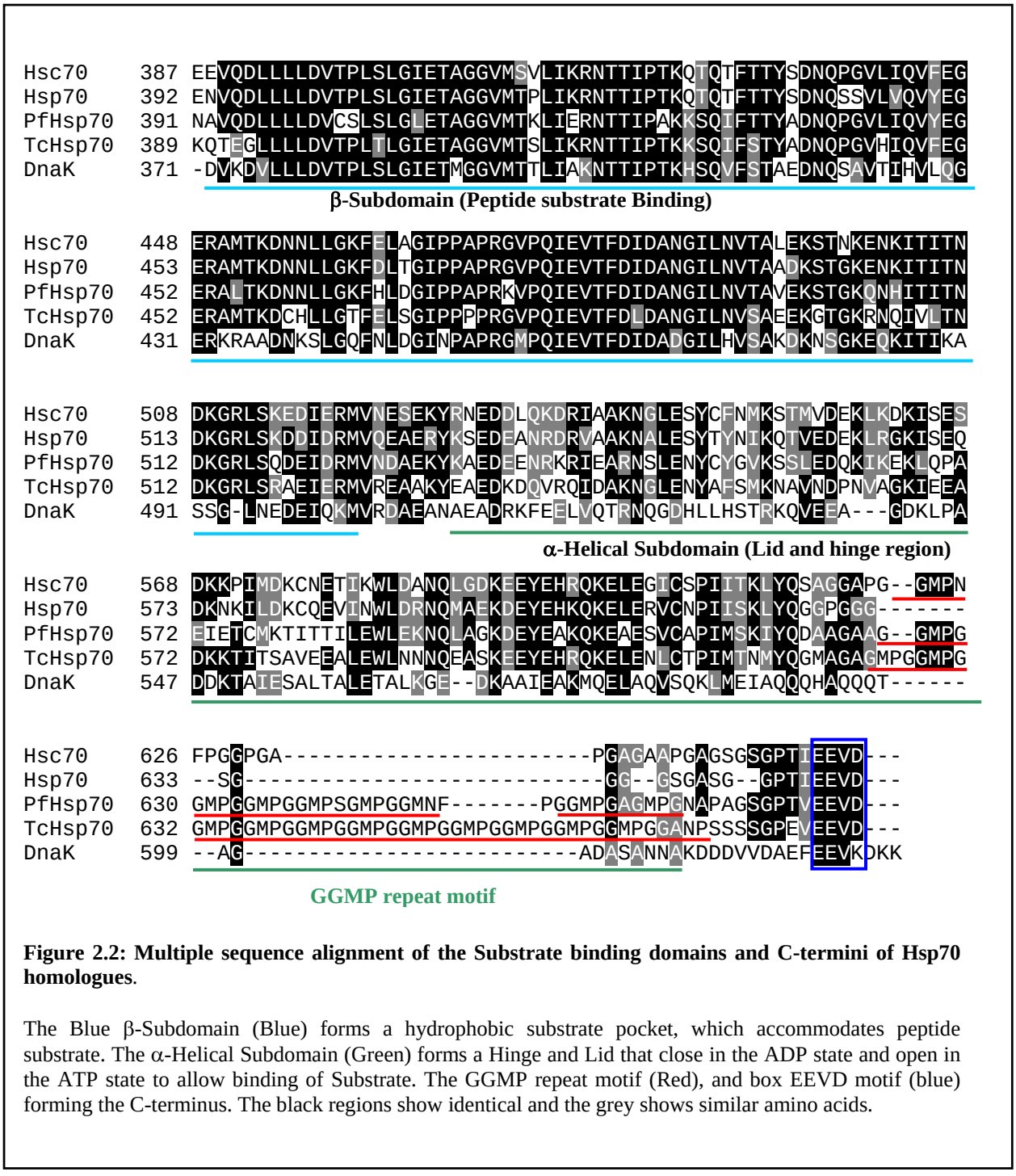


Figure 2.2: Multiple sequence alignment of the Substrate binding domains and C-termini of Hsp70 homologues.

The Blue β-Subdomain (Blue) forms a hydrophobic substrate pocket, which accommodates peptide substrate. The α-Helical Subdomain (Green) forms a Hinge and Lid that close in the ADP state and open in the ATP state to allow binding of Substrate. The GGMP repeat motif (Red), and box EEVD motif (blue) forming the C-terminus. The black regions show identical and the grey shows similar amino acids.

2.3.14 MODELLING OF PfHsp70

WHATIF was used in the modelling of PfHsp70 using Bovine Hsc70 as temple the model is depicted below. The ATPase binding cleft and Hsp40 (DnaJ) binding site are shown (Figure 2.3). PfHsp70 ATPase domain is made up of two domains A and B, which comprise of two sub-domains (IA, IIA, IB, IIB).

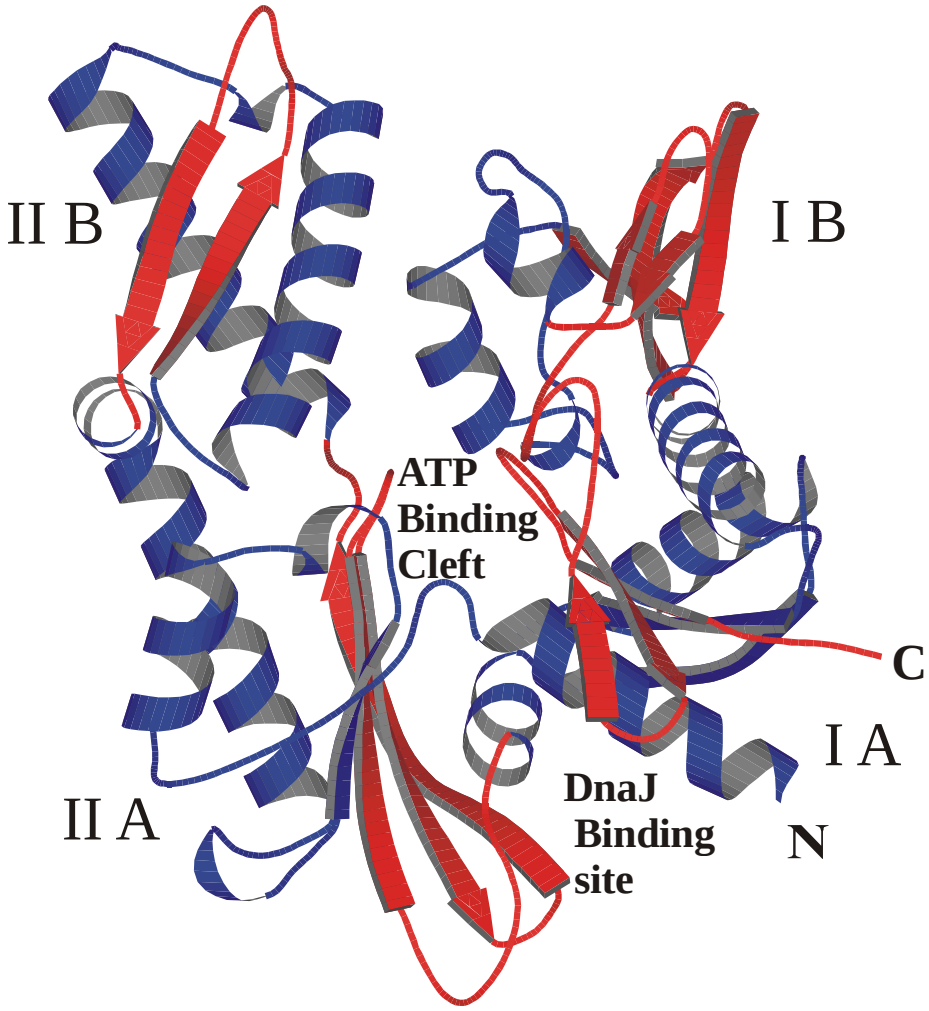


Figure 2.3: Ribbon representation of the predicted Tertiary structure of PfHsp70.

Bovine Hsc 70 (PDB 3HSC) was used as a template for the modelling of PfHsp70. The ATP binding cleft subdomain and DnaJ binding site are indicated, N (N-terminal) and C (C-terminal). The model was generated using WHATIF (Chinea *et al.*, 1996) and the coordinates visualized using Molscript (Kraulis, 1991).

2.3.2 Multiple sequence alignment of Pfjs

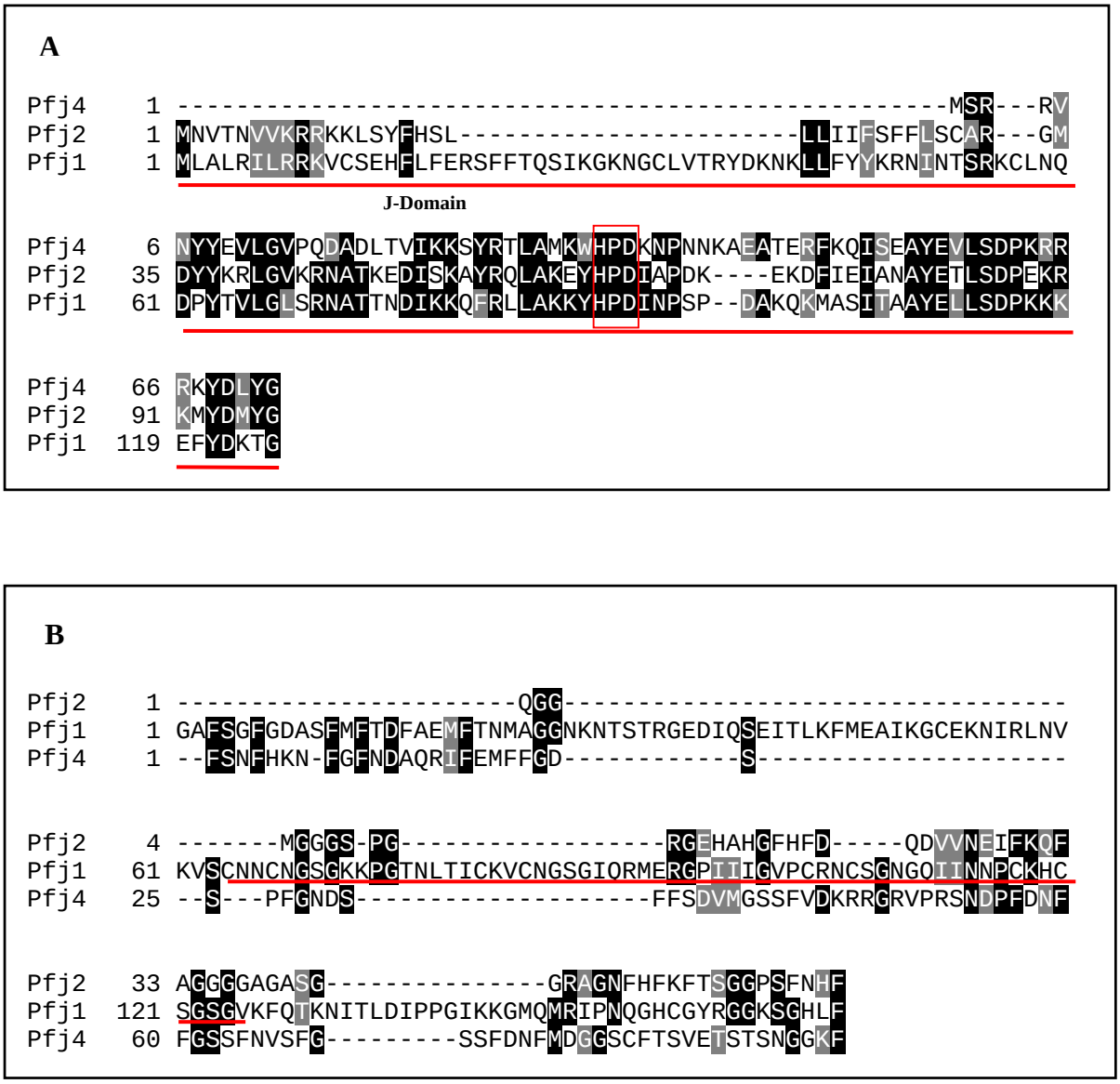


Figure 2.4: Multiple sequence alignment of Pfjs

(A): J-Domain of Pfj1-4, HPD region is indicated in red box. (B): G/F region and the Cys regions. Pfj1 is a Type I and Pfj2 and 4 are Type II DnaJ like proteins (Cheetham and Capian, 1998) proteins, Cys region is underlined in red. Type II proteins have no Cys region the only have a J-domain and G/F region where as Type I have both J-domain and G/F region and a Cys region.

Hsp40 (DnaJ) proteins are known to stimulate the ATP hydrolysis activity of Hsp70s. In *P. falciparum* there are four types of DnaJ proteins; Pfj 1-4. Full protein sequences for three of the Pfj proteins (Pfj1, 2 and 4) were obtained from Pubmed browser (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi). **A m**ultiple sequence alignment was generated using, on the three proteins in order to determine their similarities. They all contained the His-Pro-Asp (HPD) motif (Red box) which is conserved in all Hsp40 (DnaJ) proteins. Pfj1 is a Type I DnaJ protein according to the classification by Cheetham and Chaplan, 1998. This protein contains all 3 domains; a J-domain of approximately 70 amino acid at the N-terminus (underlined in red), followed by a glycine/phenylalanine (G/F) rich region (Green) and a cysteine (Cys) rich domain (Blue). Pfj2 and 4 are Type II DnaJ proteins that only contain a J-domain and a much longer G/F rich region. Very little or no work has been done on Pfj3 proteins; work is required on determining the Type and similarities it has on the other Pfjs. Alignment of the Pfj proteins reveals that they are very different with an overall percentage identity of 16 %, however the overall percentage identity of the J-domains was 37 %.

2.3.3 ALIGNMENT OF TYPE I DnaJ PROTEINS.

2.3.3.1 COMPARISON OF THE J-DOMAIN OF Hsp 40 HOMOLOGUES

Multiple sequence alignment was carried out on Type I DnaJ proteins from various organisms. Tcj2 (*Trypanosoma cruzi DnaJ 2),* Ydj1 (*Yeast DnaJ*), DnaJ (*Escherichia coli*) Scj1 (*Saccharomyces cerevisiae DnaJ*) and Pfj 1 (*Plasmodium falciparum DnaJ 1*). The above alignment is based on the N-terminus containing the J-domain. The conserved HPD motif (Red box) is found in all proteins. The J-domain is indicated by a blue line. Pfj1 and Scj1 contain a long stretch of amino acids before the start of the J-domain. The J-domains show a degree of conservation; this is noted by the black region (identical residues) and the grey region (similar residues). The J-domain is known to stimulate the ATPase activity of Hsp70 through its DnaJ binding site. DnaJ and Ydj1 both had the highest percentage identity of 28 % when compared to Pfj1.

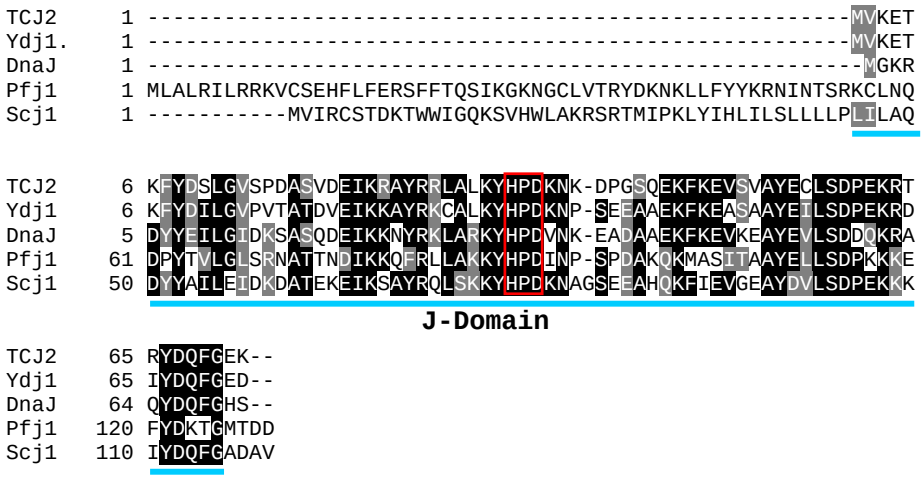


Figure 2.5: Multiple sequence alignment of the J-Domain of various Type I Hsp 40s.

Pfj 1: *Plasmodium falcipa*rum DnaJ 1 (GenBank: BAA22060);Tcj 2: *Trypanosoma cruzi* DnaJ 2 (GenBank: AAC18895), Ydj1: *Yeast* DnaJ (Gene Bank:NP_014335), Scj1: *Saccharomyces cerevisiae* DnaJ. (GeneBank: NP_013941), DnaJ: *Escherichia coli* (BAC13923) Blue [J-Domain (70 amino acid)]. Red box contains three amino acids (HPD) which are conserved in the J-Domain. The black regions show identical and the grey shows similar regions.

2.3.3.2 C-TERMINUS OF DnaJs (Hsp 40 HOMOLOGUE)

The C-terminus alignment of Type I Hsp40 proteins was carried out (Figure 2.6). The G/F region is indicated in green and the Cys region in blue. The Cys region consists of Cys-x-x-Cys-x-Gly-x-Gly (CxxCxGxG) (Blue box) where the x indicates any amino acids. As observed, Pfj1 has a C-terminus that is much longer than that of the other Type I proteins. The alignment also shows that there are very few similarities amongst the proteins these are denoted by the shadings. Yeast Hsp40 proteins contain a high G/F content than that of parasitic Hsp40 proteins. The extra amino acids found in Pfj1 may have some significant functional role within the parasite's mode of action during its stimulation of ATPase and chaperone activity within the Human host thereby assisting in the refolding of denatured proteins.

2.3.4 MODELLING OF Pfjs

WHATIF was used to model the J domain of the of Pfjs using DnaJ (PDB code 1XBL). The J domains were modelled in order to visualize the orientations using Molscript. The conserved HPD motif is indicated in red and is found in the loop between helices II and III. The

orientation of the HPD in Pfj2 has a significantly different orientation compared to that of the other J domains (Figure 2.7). Tcj2 was included in the modelling because like Pfj1 it is a Type 1 DnaJ protein, and it was used in this study as a co-chaperone to stimulate the ATPase activity of PfHsp70. Its similarities to Pfj1 were of great importance as it could be used as a substitute, since at the time of experiments purified Pfj proteins were not available but purified Tcj2 was available. Comparing the two models (Pfj1 and Tcj2) it is shown that the domains are significantly similar (Figure 2.7). Multiple alignments show that the J domains are conserved (Figure 2.5). The modelled J-domain structures only reveal a predicated orientation of the residues with the proteins.

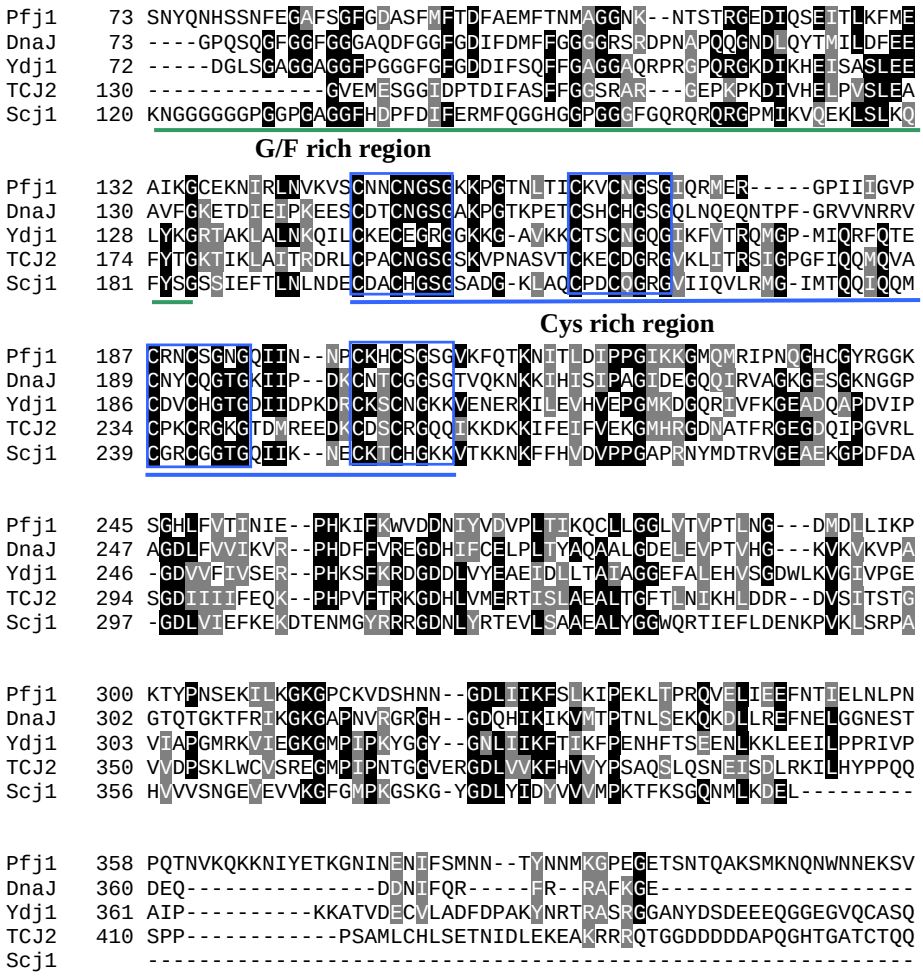
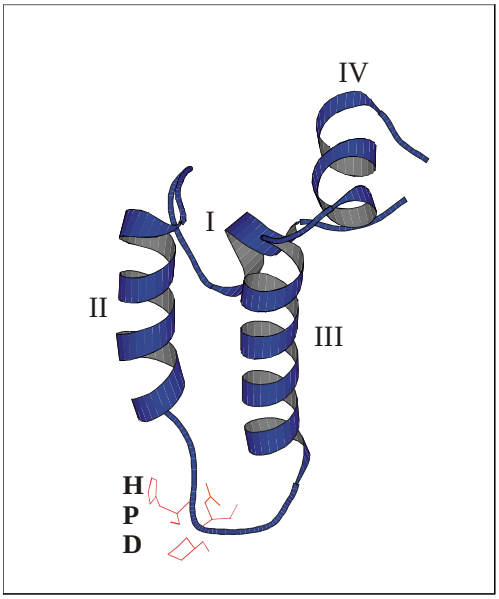
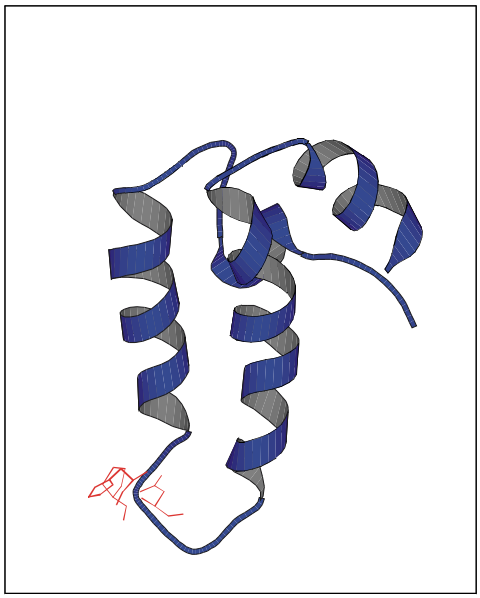


Figure 2.6: Multiple sequence alignment of the C-terminus of Type I Hsp40s from various organisms

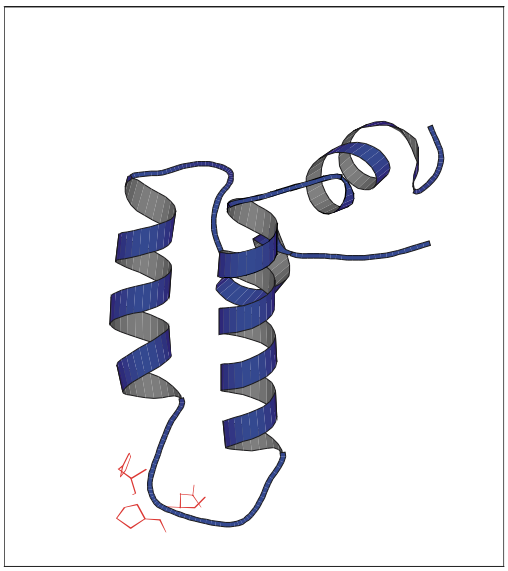
Multiple alignment of the C-terminus of Type I Hsp40s from various organisms; Pfj 1: *Plasmodium falciparum* DnaJ 1 (GenBank: BAA22060); Tcj 2: *Trypanosoma cruzi* DnaJ 2 (GenBank: AAC18895), Ydj1: *Yeast* DnaJ (GeneBank: NP_014335), Scj1: *Saccharomyces cerevisiae* DnaJ. (GeneBank: NP_013941), DnaJ: *Escherichia coli* (BAC13923). G/F rich region (Green). Cys region (Blue). The black regions show identical and the grey shows similar regions. Pfj1 has an extension of 200 amino acids longer than that of the other Hsp40 proteins, which is not shown in the above figure.



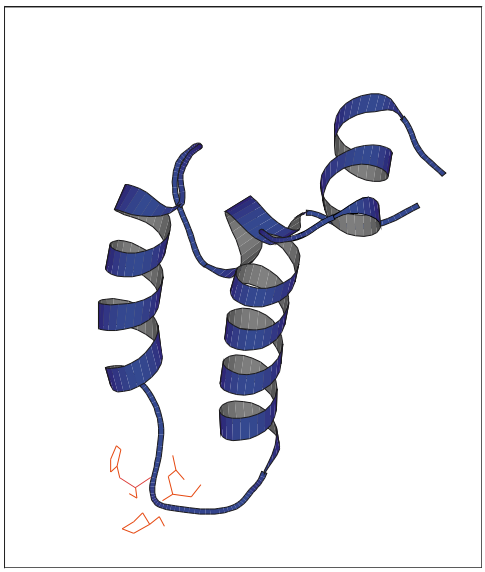
Pfj1



Pfj2



Pfj4



Tcj2

Figure 2.7 Ribbon representation of J-domains of Pfjs and Tcj2.

The ribbon representation of the J-domains of *Plasmodium falciparum* DnaJs 1,2,and 4 and *Trypanosoma cruzi* DnaJ 2 are shown above. The conserved HPD residues are indicated in red. The models were generated using WHATIF (Chinea *et al*., 1996) and visualized using Molscript (Kraulis,1991).

2.4 CONCLUSION

Multiple alignment of PfHsp70 with other Hsp70s from various organisms revealed PfHsp70 to have an N-terminal ATPase domain, central substrate domain and a C-terminal domain. Like other parasitic Hsp70s a GGMP motif was observed in PfHsp70, this motif is known to induce immune response in its Host. An EEVD motif was also observed for PfHsp70 this motif is found in eukaryotic Hsp70s and is known to bind to TPR domain of HOP during folding of protein. A 70 % identity Between PfHsp70 and Human Hsp70 of known crystal structure was observed, as a result a predicted structure for PfHsp70 was generated using WHATIF and visualized using Molscript.

P. falciparum has four DnaJs, which have an overall percentage identity of 16 %. A 37 % Identity was observed for the J domains, this revealed that the J proteins were very different. Pfj 1, which is a Type I had a 28 % identity to other Type I DnaJ proteins from various organisms. Homology modelling of the predicated structures of the J domains revealed the three Pfjs to have different orientations. Full sequence of Pfj3 was not available at the time of experiments.

CHAPTER 3

HETEROLOGOUS PRODUCTION, PURIFICATION AND BIOPHYSICAL CHARACTERIZATION OF PfHsp 70

3.1 INTRODUCTION

3.1.1 HETEROLOGOUS PROTEIN PRODUCTION

E. coli is a popular host for the heterologous expression of proteins (Kane, 1995). When expression is induced by the addition of inducing agent such as isopropyl-beta-D- thio-galactopyranoside (IPTG), large quantities of heterologous mRNA are produced. *E. coli* uses a specific subset of the 61 amino acid codons for the production of most mRNA molecules (Kane, 1995). It would not be problematic if the mRNA were to contain a distribution of codons for the protein or a distribution of amino acids that *E. coli* normally encounters, however if the heterologous mRNA were to contain rare codons or if the amino acid distributions were inordinately skewed relative to *E. coli* proteins, then it is likely that translational problems would occur during the production phase leading to a reduction in either the quantity or quality of the protein synthesized (Kane, 1995).

Overexpression of parasite genes is important for providing a sufficient amount of recombinant protein for characterization. Overexpression of *P. falciparum* genes in *E. coli* has been difficult because of the codon bias of the organisms (Baca and Hol, 2000). *P. falciparum* contains an extremely high A-T rich genome of about 80 % resulting in rare codon usage in *E. coli.* In *E.coli* mRNAs, containing codons for arginine (AGG or AGA), glycine (GGA) and isoleucine (AUA), were found to be rarely used and had a detrimental effect on protein expression (Kane, 1995, Baca and Hol, 2000). The rare codons appeared to cause problems in translation causing translational errors such as aborted translation. The recommendation would be to examine the sequence of the heterologous gene for these codons and then either convert them to commonly used codons or use a host containing a plasmid with the appropriate tRNA molecules for the rare codons (Kane, 1995).

3.1.2 PROTEIN PURIFICATION

Development of various bacterial vectors based on affinity tag purification systems has revolutionized the field of biological sciences (Smith and Johnson, 1988). The polypeptide is made as a fusion with an affinity tag that allows its purification in a single step. A commonly used affinity tag is the glutathione *S*-transferase (GST) fusion system (Smith and Johnson, 1988). The desired polypeptide is made in frame as a GST fusion and the fusion protein is purified by glutathione agarose chromatography in a single step (Smith and Johnson, 1988).

The presence of specific protease recogniton sites in the fusion protein allows for the release of the affinity tag from the desired peptide, which is especially usful when using large tags such as GST (Smith and Johnson, 1988). Another widely used expression system is based on the use of the histidine affinity tag (6xHis), which allows for the purification of expressed proteins on a Ni-containing matrix (Gentz *et al*., 1989). The most commonly used chromatography matrix is the nickel-nitrilotriactetic acid (Ni-NTA) metal-affinity chromatography matrices based on the nickel ions binding to the 6xHis-tag contained within the protein of interest. Imidazole is used; to elute the ptotein and it has a similar structure to that of histidine. Imidazole itself can also bind to the nickel ions and disrupt the binding of dispersed histidine residues in untagged background proteins (Hochuli *et al*., 1987). Since the interaction between Ni-NTA and 6xHis-tag of the recombinant protein does not depend on tertiary structure, proteins can be purified under either native or denaturing conditions. To set up the best purification strategy, it is important to determine whether the protein is soluble in the cytoplasm or located in cytoplasmic inclusion bodies (The QIA expressionist, 1999).

3.1.3 OBJECTIVE

Plamodium genome has a rich A-T content of about 80%. This posses a major problem in expressing and purifying in *E. coli* host, which is more biased towards G-C usage. The broad objective of this study was to improve heterologous over-production and purification of PfHsp70 in *E. coli.* The coding region was amplified by PCR and inserted into a suitable expression vector. Over-production of the protein was achieved by inducing cells with IPTG, and the degree of over production was analysed by Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western analysis. Solubility studies were used to determine the purification approach. If the protein was soluble then purification would be carried out under native conditions but if the protein was insoluble then a denaturing purification using 8M urea would be carried out. Molecular Weight determination of PfHsp70 was carried out using size exclusion on a High performance liquid chromatography (SE-HPLC). Complex formation in the presence of substrate was determined using reduced and carboxymthylated lactalbumin (RCMLA) as substrate.

3.2 EXPERIMENTAL METHODOLOGY

3.2.1 MATERIALS

All reagents were obtained from Sigma Chemicals Co. (St.Louis,Mo U.S.A.), Roche Molecular Biochemicals (Indianapolis, IN, U.S.A.) or Merck Chemicals(Darmstadt, Germany) unless stated otherwise. Hybond™Cextra chemiluminescence nitrocellulose was purchased from Amersham Phamacia Biotech (Piscataway, NJ, U.S.A). pQE30 plasmid was purchased from Qiagen (La Jolla, CA, U.S.A.). pRSETb/PfHsp70 plasmid was generated by Dr.N.Kumar (Department of Microbiology and Immunology,John Hopkins University,USA). Nickel-chelating Sepharose Fast Flow was obtained from Pharacia Biotech (Uppsala, Sweden). RIG plasmid was generated by Professor W.G.J.Hol (Biomolecular Structure Center, University of Washington, U.S.A). pGEM®-T Easy Vector Systems Promega (Germay), *Hind*III and *Bam*HI restriction enzymes were purchased from Amersham Phamacia Biotech (Piscataway, NJ, U.S.A). Monoclonal Mouse anti-His antibody was purchased from, Amersham pharacia biotech,UK.

3.2.2 CULTURING OF *E.coli*

3.2.2.1 *Culturing of E.coli BL21 (DE3) (pLysS)[pRSETb/PfHsp70]*

2x YT broth (2x Yeast-Tryptone) (1.6 g tryptone, 1.0 g yeast, 0.5 g NaCl) and 2x YT broth agar (1.6 g tryptone, 1.0 g yeast, 0.5 g NaCl, 1.5 g agar) plates were prepared and autoclaved. Ampicillin (AMP) and chloramphenicol (CAM) were added to both broth and plates to 100 mg/ml and 34 mg/ml final concentrations respectively. Single colonies of *E. coli* BL21 (DE3) (pLysS)[pRSETb/PfHsp70] from 2x YT broth AMP/CAM agar plates were cultured in 5 ml 2x YT broth AMP/CAM and incubated at 37˚C for 16 hours with shaking (250 rpm).

3.2.3 CONFIRMATION OF IDENTITY OF PfHsp70 cDNA IN pRSETb VECTOR

3.2.3.1 *Small scale plasmid DNA preparation*

The plasmid pRSETb/PfHsp70 was a kind donation from Dr.N.Kumar (*Department of Microbiology and Immunology, John Hopkins University, USA*). 1 ml of overnight

E.coli BL21 (DE3) (pLysS) [pRSETb/PfHsp70] culture was centrifuged at 16 000 x g for 1 minute, and the cell pellet resuspended in 100 µl of solution I (50 mM glucose; 25 mM Tris-Cl, pH 8.0; 10 mM EDTA, pH 8.0). 200 µl solution II (0.2 M NaOH, 1% SDS) was then added and the suspension incubated on ice. After 2 minutes, 150 µl of solution III (3 M potassium acetate pH 5,0) was added, the suspension mixed gently by inversion and incubated on ice for 5 minutes. The genomic DNA and cell debris was pelleted by centrifugation at room temperature for 10 minutes. The supernatant was transferred into a new sterile tube. The plasmid DNA was precipitated by the addition of 450 µl isopropanol and incubated at room temperature for 2 minutes. The plasmid DNA precipitate was then collected by centrifugation at 4˚C for 20 minutes, air dried, and resuspended in 400 µl TE buffer (Tris-EDTA, 10 mM Tris-Cl, 1 mM EDTA). 40 µl of 3 M sodium acetate (pH 5.2) was then added, followed by the addition of 800 µl of ice-cold absolute ethanol. The suspension was incubated at –20˚C for 1 hour. The suspension was centrifuged as before, the pellet washed by the addition of 200 µl 70% ethanol and re-centrifuged for 1 minute. The final plasmid DNA pellet was air-dried, resuspended in 25 µl TE buffer, and stored at -20˚C.

3.2.3.2 *Restriction endonuclease digestion and agarose gel analysis of pRSETb/PfHsp70.*

The identity of pRSETb/PfHsp70 plasmid DNA was confirmed by digestion with *Hind*III and *Bam*HI. pRSETb/PfHsp70 plasmid DNA (2 x 1 µl) was diluted into 17 µl of sterile deionised milli Q water. 10x restriction buffer A (2 µl) (100 mM Tris –HCl, pH 7.5, 100 mM $MgCl_2$, 10 mM Dithiothreitol, 500 mM NaCl) and 2 units of *Hind*III and 10x restriction buffer B (2 µl) (100 mM Tris-HCl, pH 8.5, 100 mM $MgCl_2$, 10 mM Dithiothreitol, 1 M KCl) and 2 units of *Bam*HI were added to respective DNA to be digested. The digestion reaction was incubated overnight at 37˚C. A double digest reaction whereby both *Hind*III and *Bam*HI were added was also performed. In this case *Hind*III was added first, the reaction incubated for 3 hours before the addition of *Bam*HI and incubation as before.

A 0.8 % agarose gel was prepared in TBE buffer (Tris Borate EDTA: 0.045 M Tris, 0.045 M Borate, 0.001M EDTA) containing ethidium bromide (10 mg/ml). A sample of each restriction endonuclease digestion (10 µl) was analysed. lambda (λ) DNA cut

with *Pst*I was used as marker (marker sizes ranged from 1,093-11,479 bp). The gel was electrophoresed at 100V for 60 minutes.

3.2.4 HETEROLOGUS PRODUCTION OF RECOMBINANT $(His)_6$-PfHsp70

Overnight *E.coli* BL21 (DE3) (pLysS)[pRSETb/PfHsp70] and *E.coli* BL21 (DE3) (pLysS) [pQE30/PfHsp70] cultures were diluted into 25 ml 2x YT broth containing AMP/CAM. Diluted cultures were then grown at 37˚C until absorbance at 600 nm reached 0.6 - 0.9 units. Samples (2 ml) were removed from the cultures for SDS-PAGE analysis. $(His)_6$-PfHsp70 expression was then induced by the addition of isopropyl-beta-D- thio-galactopyranoside (IPTG) to a final concentration of 1 mM, and the incubation of the cultures resumed. Samples (2 ml) were subsequently removed every hour after induction, for 5 hours. A final sample after 16 hrs was also taken. The induced cell pellets were collected by centrifugation at 16 000 x g at room temperature for 60 seconds. The cells were resuspended immediately in ice cold PBS (phosphate buffered saline; 0.13 M NaCl, 2,68 mM KCl, 0.01 M Na_2HPO4, 1.76 mM KH_2PO4, dissolved in 1 L distilled water, pH 7.4) at a ratio of 50 µl per hour induction in order that cell densities were made uniform. The induced cell suspensions were stored at –20˚C until all samples were ready for Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS PAGE) analysis.

A 2x Sodium dodecylsulphate (SDS) gel loading buffer (100 mM Tris, pH 6.8 ; 5 % β-mercaptoethanol; 4 % SDS; 0.2 % bromophenol blue and 20% glycerol) was diluted into each induced cell suspension and incubated for 3-5 minutes at 100˚C. The tubes were briefly vortexed and 20 µl of each sample separated on a (0.1 %) SDS (12 %) PAGE gel electrophoresed in Tris-glycine buffer (pH 8.3)(0.12 M Tris-base and 1.26 M glycine). SDS-PAGE markers (Myosin, 200 kDa; β-galactosidase, 116.3 kDa; Phosphorylase b, 97.4 kDa; Bovine serum albumin, 66.2 kDa; Ovalbumin, 45 kDa; Carbonic anhydrase, 31 kDa; Soybean trypsin inhibitor, 21.5 kDa; Lysozyme, 14.6 kDa; and Aprotinin, 6.5 kDa)(Biorad catalogue number 161-0317) were used to determine protein size. The gel was electrophoresed at 200 V for 45 minutes, stained in gel staining solution (0.24 % Coomassie blue, 50 % Methanol, 7.5 % Acetic acid)

for 3 hours, and destained in destaining solution (20 % methanol, 7.5 % Acetic acid) overnight.

3.2.5 WESTERN ANALYSIS

Cell lysates of induced cells were separated by (0.1 %) SDS (12 %) PAGE as before, and the gel pre-equilibrated in Western transfer buffer (39 mM glycine, 48 mM Tris-base, 20 % Methanol). A Western blot membrane (Hybond-Cextra, Amersham,U.S.A) Cat.No.RPN203E) was prepared by placing a nitrocellulose membrane against the (0.1 %) SDS (12 %) PAGE gel, flanking both with pre-eqilibrated 3 MM paper and sponge pads, and transferring proteins onto the nitrocellulose (towards the cathode) at 100 V for one hour. The nitrocellulose was stained with Ponceau S stain (0.5 % Ponceau S, 1 % glacial acetic acid) for 2 minutes, and destained with distilled water. The membrane was then washed twice with TBS (Tris buffered saline: 50 mM Tris, 150 mM sodium chloride, pH 7.5) and blocked with 5% block (5 % non-fat milk in TBS) with shaking for one hour. The membrane was incubated with anti-His primary antibody (monoclonal Mouse ascites fluid in 0.1 % sodium azide, Amersham pharacia biotech,UK), diluted in 5 % block (2 µl in 6 µl block), for one hour. The membrane was then washed in TBST (1 % Tween 20 in 1 litre TBS) for 20 minutes and twice in 5% block for 20 minutes. The membrane was incubated with secondary antibody ((4 µl in 5 ml block) (anti-mouse IgG-POD/anti-rabbit IgG-POD, Roche, Germany) for 30 minutes. The membrane was washed four times with TBST for 15 minutes each. The chemiluminescence detection reagents were prepared (60 µl starting solution added to 6 ml luminescence substrate solution at 25˚C and incubated at room temperature for 60 seconds). The detection reagent were added to the blot, incubated for 60 seconds, drained and wrapped in cling-wrap before exposure to X-ray film (Kodak) for varying times of 10 seconds – 2 minutes. The film was developed in developer (G138i, AGFA) for 2 minutes, passed through stop solution (2 % acetic acid) and fixed in fixer (G 334i, AGFA) for 2 minutes.

3.2.6 PfHsp70 CONSTRUCT

3.2.6.1 *Preparation of competent cells*

Overnight *E.coli* BL21 (DE3) culture was diluted (1:20) into 25 ml 2x YT broth containing no antibiotics. Diluted cultures were then grown at 37˚C until absorbance at 600nm reached early log phase (0.3 - 0.6 units). The cells were harvested by centrifuging at

4 500 x g for 15 minutes at 4 ºC (in sterile centrifuge tubes). Cells were kept on ice at all times for the rest of the preparation. Cells were resuspended in 25 ml of ice cold 0.1 M $MgCl_2$ and left on ice for 20 minutes. Cells were then collected as above by centrifuging for 15 minutes and resuspended in 12.5 mL of ice cold 0.1 M $CaCl_2$ and left for 1 hour. Cells were collected and gently resuspended in 2.5 ml of ice cold 0.1 M $CaCl_2$. For storage the cells were put into microcentrifuge tubes and 1 volume of ice cold sterile glycerol was added and stored at -70˚C.

3.2.6.2 *Polymerase chain reaction of PfHsp70*

Polymerase chain reaction (PCR) was used to isolate PfHsp70 coding region from pRSETb/PfHsp70 plasmid using Forward primer (´5-GCGGATCC ATGGCTAGTG CAAAAGGTT-3´)(Annealing temperature 65 ºC) and Reverse primer (´5-CGAAG CTTTTAATCAACTTCTTCAACTG-3´)(Annealing temperature 57 ºC). 2 separate master mixes were prepared according to the protocol for the expand High Fidelity PCR system (Roche, Germany). In one PCR tube 1 µl of dNTP mix (10 mM Deoxyribonucleoside Triphosphate), 5 µl each of Forward primer (15 µM) and Reverse primer (15 µM), 2 µl of template DNA (200 $ng.\mu l^{-1}$ PfHsp70) and ultrapure distilled water (ddd H_2O) was added to a final volume of 25 µl. In a separate PCR tube 5 µl of 10x Expand High Fidelity buffer (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 1 mM dithiothreitol, 0.1 mM EDTA, 0.5% Tween, 0.5% Nonidet, 50% glycerol with 15 mM $MgCl_2$) , 1 µl of Expand High Fidelity PCR enzyme mix (3.5 U) and ddd H_2O was added to a final volume of 25 µl. The two master mixes were mixed, and subjected to the following protocol in a Thermo-cycler, Stage 1, 1 x cycle at 94 ºC for 2 minutes; Stage 2, 30 x cycles at [94 ºC for 30 seconds, 55 ºC for 1 minute, 72 ºC for 2,5 minutes]; Stage 3, 1 x cycle at 72 ºC for 10 minutes.

3.2.6.3 *Inserting into pQE30 vector via pGEM-T easy vector*

Amplified product corresponding to fragment 2 045 bp (base pairs) was ligated into pGEM-T Easy vector (Promega, Germany). Rapid ligation Buffer (2x; 5 µl), 1 µl of pGEM-T Easy Vector (50 ng/µl), amplified fragment (200 ng; 3 µl), T4 DNA ligase (3 Weiss units/µl; 1 µl) were mixed and incubated overnight at 4 ˚C, sample of the ligation reaction (5 µl) was transferred to 100 µl competent cells (*E. coli* XL1 Blue) then stored on ice for 20 minutes. Heat-shock was carried out for 50 seconds in a water bath at 42 ˚C. Cells were left on ice for 2 minutes. 2xYT broth (900 µl) was added and the mixture incubated for 90 minutes with shaking at 37 ˚C (250 rpm). Screening was carried out on 100 µl transformation culture, on Lactose Macconkey Agar plates (20 gL^{-1} Peptone, 10 gL^{-1} Lactose, 1.5 gL^{-1} Bile Salt No.3, 5 gL^{-1} Sodium chloride, 0.03 gL^{-1} Neutral, 0.001 gL^{-1} Crystal violet, 13.5 gL^{-1} Agar) containing 100 mg/ml AMP. Plasmid DNA was isolated (see section 3.2.3.1) and digested with *Bam* HI and *Hind* III (See section 3.2.3.2). The released insert was ligated into *Bam* HI / *Hind* III digested pQE30 expression vector to generate plasmid pQE30-PfHsp70.

3.2.7 ENGINEERING OF RIG PLASMID INTO *E.coli*

The RIG plasmid contains genes that express for tRNAs; arginine (AGG or AGA) , glycine (GGA) and Isoleucine (AUA) which are reare in *E. coli* (Hol, 2000). The RIG Plasmid was transformed into *E. coli* XL1 Blue together with pQE30/PfHsp70 construct as above.

3.2.8 SOLUBILITY STUDIES OF RECOMBINANT $(His)_6$-PfHsp70

$(His)_6$-PfHsp70 was produced in *E.coli* (XL1 Blue) as before. Pre-warmed 2x YT broth (50 ml) with AMP (100 mg/ml) was innoculated with 5 ml overnight *E.coli* (XL1 Blue)[pQE30/PfHsp70] cultures, and grown at 37˚C, with shaking until absorbance at 600 nm reached 0.5 - 0.7 units. Samples (2 ml) were removed from the cultures for SDS-PAGE analysis. $(His)_6$-PfHsp70 expression was then induced by the addition of IPTG to a final concentration of 1 mM, and the incubation of the cultures resumed for 4 hours at 37 ˚C. The induced cells were collected by centrifugation at 4

500 x g for 20 minutes, and resuspended in 5 ml lysis buffer (0.01 mM Tri, pH 8.0, 300 mM NaCl, 10 mM imidazole, 1 mM phenylmethylsulfonyl fluoride). Lysozyme was added to a final concentration of 1 mM in order to disrupt the cell wall. The induced cells were then frozen at –70˚C overnight, thawed in water, and centrifuged at 16 000 x g at 4˚C for 20 minutes. The supernatant was decanted (crude extract A, soluble protein), and the pellet was resuspended in 5 ml PBS buffer (crude extract B, insoluble protein). Samples were analysed on a (0.1 %) SDS (12 %) PAGE gel.

3.2.9 BATCH PURIFICATION OF RECOMBINANT $(His)_6$-PfHsp70

E.coli XL1 Blue [pQE30/PfHsp70] was cultured (1 Litre) and production of $(His)_6$-PfHsp70 was induced by the addition of IPTG as before (section 3.2.4). Induced cells were harvested by centrifugation at 4 500 x g for 20 minutes at 4˚C. The induced cell pellet was resuspended in 10 ml denaturing lysis buffer (8 M urea, 300 mM NaCl, 10 mM imidazole, 0.01 M Tris, pH 8.0,) 1 mM final concentration of phenylmethylsulfonyl fluoride (PMSF) was added to inhibit protease action and Lysozyme was also added to a final concentration of 1 mM. Induced cells (10 ml) were lysed by freeze/thaw and soluble protein extract prepared as before (section 3.2.8) . Ni-chelating sepharose resin slurry 50 %; 2 ml; (Chelating sepharose® Fast flow, Pharmacia Biotech, Sweden) was added to the 4 ml clear soluble protein extract and mixed gently by agitation at 4˚C for 60 minutes. The lysate- Ni-chelating sepharose beads were centrifuged at 16 000 x g for 5 minutes in order to remove unbound protein. The supernatant (flow-through) was collected. Ni-chelating sepharose beads were washed twice by gently agitating for 5 minutes at 4˚C with wash buffer (300 mM NaCl, 50 mM imidazole 0.01 M Tris, pH 8.0) then centrifuging at 16 000 x g for 5 minutes. Two more washes were carried out uising increasing concentrations of imidazole (100 mM imidazole, 300 mM NaCl, 0.01 M Tris, pH 8.0). The elution of PfHsp70 was repeated 4 times as above with 0.5 ml elution buffer (300 mM NaCl, 0.01 M Tris pH 8.0, 1 M imidazole). Eluants were collected and analysed by (0.1 %) SDS (12 %) PAGE.

3.2.10 PROTEIN CONCENTRATION ANAYSIS

Protein concentration was determined by Bradford's method (Bradford, 1976). A BSA standard was set up using concentrations from 0 to 200 μg/ml in 0.15 M NaCl as in table A2 (Appendix A), were prepared. Bradford's reagent (250 μl) (10 % Coomassie G250 in 95% ethanol, 85 % (w/v) phosphoric acid) was added to 5 μl of protein and the reaction incubated at room temperature for 5 minutes. Absorbance was read at 595 nm. PfHsp70 eluants (5 μl) were similarly treated, and the protein concentration determined by extrapolation from the standard curve (Appendix A4).

3.2.11 SE-HPLC ANALYSIS

Gel filtration chromatography of PfHsp70 (concentration used ranged between 10 – 18 μM) was performed using an ÄKTA*design* system FPLC/HPLC instrument using Superdex™ 200 HR 10/30 column (10 mm x 30-31 cm; bed volume of 24 ml; Amersham Pharmacia Biotech, Sweden) at 25 °C with 50 mM Tris, 0.15 M NaCl as the mobile phase; the flow rate was 0.5 ml/min. Elution volumes of 1 ml were collected. Detection was by absorbance at 280 nm. All samples were filtered through a 0.22 μM filter before injecting on the SE-HPLC. Molecular weight values were compared with a calibration curve using β-Amylase (200 kDa), Ovalbumin (45 kDa), Alcohol dehydrogenase (150 kDa), β-Galactosidase (116 kDa), BSA (66 kDa), Ovalbumin (45 kDa) as standards.

3.2.12 ANALYSIS OF COMPLEX FORMATION

3.2.12.1 *Protein substrate binding assay*

PfHsp70 (final concentration of 14 μM) was pre-incubated in Buffer B (20 mM Hepes, 5 mM $MgCl_2$ 100 mM NaCl pH 7.2) for 5 minutes at 37 ºC. RCMLA (final concentration of 40 μM) was added to the reaction in a final volume of 20 μl and allowed to bind to PfHsp70 for 30 minutes at 37ºC. Native gel loading buffer 5 μl (100 mM Tris, pH 6.8; 0.2 % bromophenol blue and 20% glycerol) was added to the reaction mixture and a sample (25 μl) immediately loaded onto a 10% native PAGE gel, electrophoresed in Tris-glycine buffer (pH 9.0) (0.12 M Tris-base and 1.26 M glycine). The gel was electrophoresed at 200 V for 45 minutes, stained in gel staining solution (0.24 % Coomassie blue, 50 % Methanol, 7.5 % Acetic acid) for 3 hours, and destained in destaining solution (20 % methanol, 7.5 % Acetic acid) overnight.

3.2.12.2 *SE-HPLC Analysis*

PfHsp70 stock solution (40 μl; 18 μM) was mixed with RCMLA stock solution of (20 μl; 40 μM) in Buffer B (20 mM Hepes, 5 mM $MgCl_2$, 100 mM NaCl, pH 7.2) made up to a final volume of 100 μl. The reaction was incubated at 37 °C for 30 minutes and injected on the SE-HPLC instrument. Fraction volumes of 1 ml were collected at a rate of 1 ml/0.5 mins. Controls included were PfHsp70 (40 μl; 18 μM) alone and RCMLA (20 μl; 40 μM) alone.

3.3 RESULTS AND DISCUSSION

3.3.1 CONFIRMATION OF THE IDENTITY OF PfHsp70 cDNA IN pRSETb VECTOR

The plasmid pRSETb/PfHsp70 construct provided was problematic in that over-production of $(His)_6$-PfHsp70 showed no over- production of protein over a time period (Figure 3.3A). This indicated that the $(His)_6$-Tag was either hidden within the protein when folding to form a functional protein or the tag was located far from the start of the coding region. DNA sequencing analysis carried out near the start codon revealed the presence of a linker region encoding 39 amino acids. Some of these were large hydrophobic amino acids, which could have interfered with the expression of $(His)_6$-PfHsp70. To overcome this problem the coding region of PfHsp70 was isolated by Polymerase chain reaction (PCR) (see Appendix A1) and inserted into a

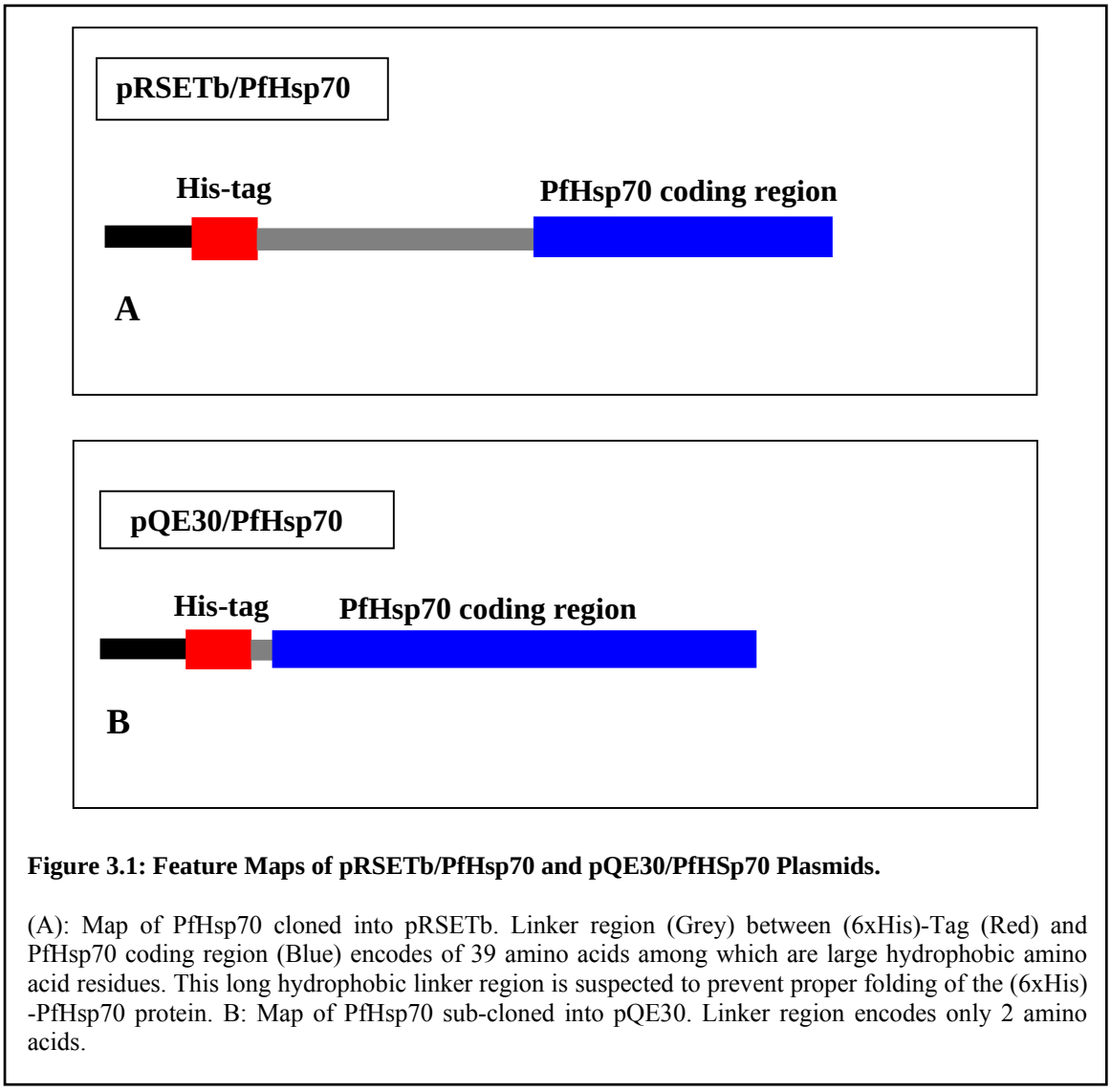


Figure 3.1: Feature Maps of pRSETb/PfHsp70 and pQE30/PfHSp70 Plasmids.

(A): Map of PfHsp70 cloned into pRSETb. Linker region (Grey) between (6xHis)-Tag (Red) and PfHsp70 coding region (Blue) encodes of 39 amino acids among which are large hydrophobic amino acid residues. This long hydrophobic linker region is suspected to prevent proper folding of the (6xHis) -PfHsp70 protein. B: Map of PfHsp70 sub-cloned into pQE30. Linker region encodes only 2 amino acids.

suitable expression vector (pQE30 expression vector) via pGEM-T easy vector (see Appendix A1and A2). Plasmid pQE30/PfHsp70 contained only 2 amino acids between the $(His)_6$-Tag and PfHsp70 start codon.

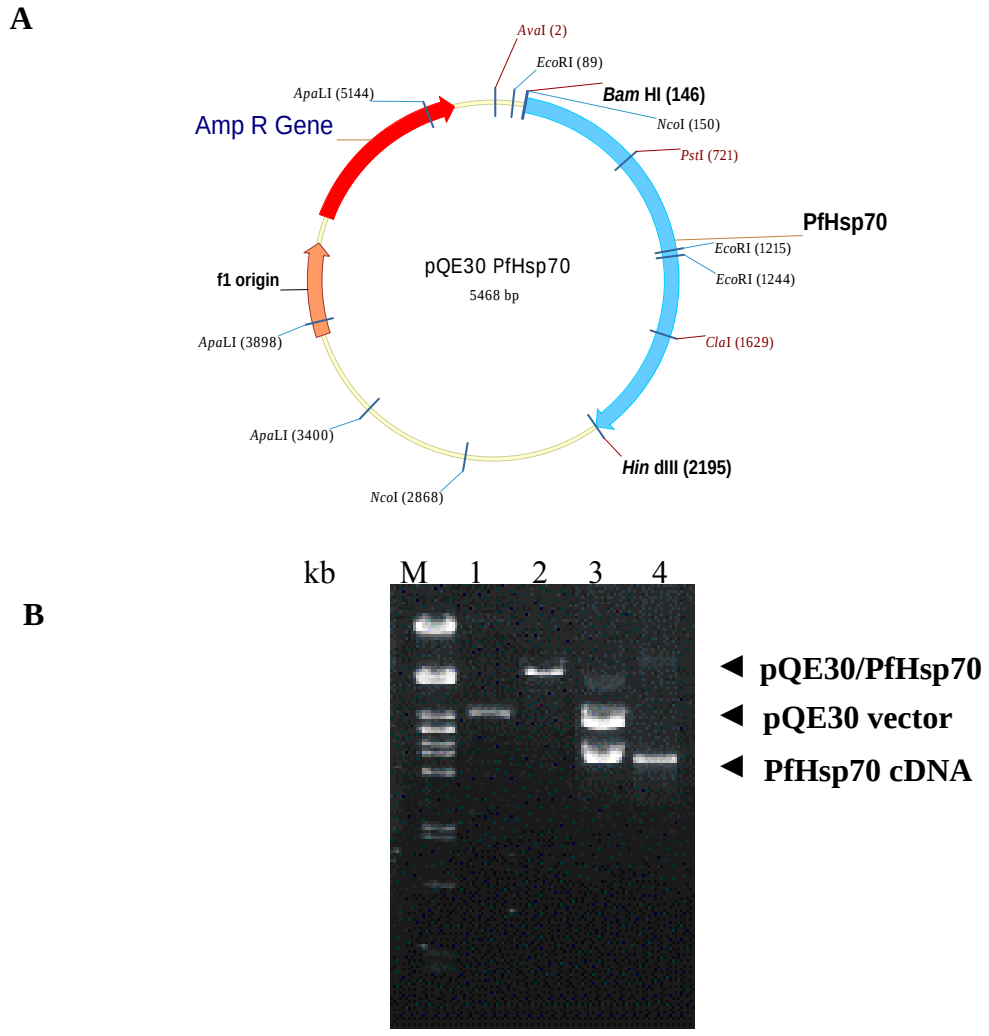


Figure 3.2: Confirmation of insertion of PfHsp70 cDNA into pQE30 vector.

(A) Map of pQE30/PfHsp70, showing PfHsp70 cDNA (blue), ampicillin resistant gene (red), *BamH*I and *Hind*III restriction sites are shown, which cut, outside the coding region. (B) Restriction enzyme digestion of pQE30/PfHsp70 plasmid construct containing the cDNA encoding PfHsp70.The plasmid was digested with *BamH*I and *Hind*III, which have sites outside the coding region. M: Molecular mass markers; Lane 1: pQE30 vector; lanes 2: pQE30/PfHsp70 linearised with *BamH*I; lane 3: pQE30/PfHsp70 double-digested with *BamH*I and *Hind*III to release the vector (pQE30, upper band) and cDNA for PfHsp70 (lower band); and lane 5: PfHsp70 cDNA PCR product.

Figures 3.3 A and 3.3 B are plasmid maps of pQE30/PfHsp70 construct showing the position of the start codon, $(His)_6$-Tag and enzyme restriction sites of *Bam*HI and *Hind*III. (C) Shows an agarose gel confirming the construct pQE30/PfHsp70. A single and double digestion of the plasmid was carried out using *Bam*HI and *Hind*III, which linearizes and releases the PfHsp70 coding region respectively. The gel therefore confirmed the construct to be pQE30/PfHsp70 plasmid and revealed the presence of the PfHsp70 conding region. DNA sequencing carried out on the pQE30/PfHsp70 construct revealed the start codon of Pfhsp70 to be only 2 amino acids upstream of the 6xHis-tag.

3.3.2 HETEROLOGUS PRODUCTION OF RECOMBINANT $(His)_6$-PfHsp70

Heterologous production of $(His)_6$-PfHsp70 in pRSETb was performed in *E.coli* (BL21)((DE3))(pLySs). As seen in figure 3.3A production of PfHsp70 was low or negligible probably due to the linker region of 39 amino acid between the $(His)_6$-tag and the start codon of PfHsp70 (Figure 3.1A). Heterologous production in the pQE30 vector (Figure 3.3B) also performed in *E.coli* BL21 (DE3) (pLySs) showed over-production with the over-night (16 hours) fraction having the highest amount of protein produced. This could probably be the result of the $(His)_6$-tag and the start codon having only 2 amino acids between them (Figure 3.1B). However multiple bands were detected by chemiluminescence–based Western analysis (Figure 3.3D). These bands are either the result of degradation or aborted translation products. *Plasmodium falciparum* has a high A-T rich genome of about 80% whereas *E.coli* is more biased towards a G-C rich genome having differing codon usage, therefore resulting in degradation or aborted translation products. There are two ways of over-coming aborted translation products; (i) to change the rare codons to suit those used by the *E.coli* host, or (ii) to engineer an *E.coli* strain by introducing a plasmid that produces tRNAs that encode for the rare codons.

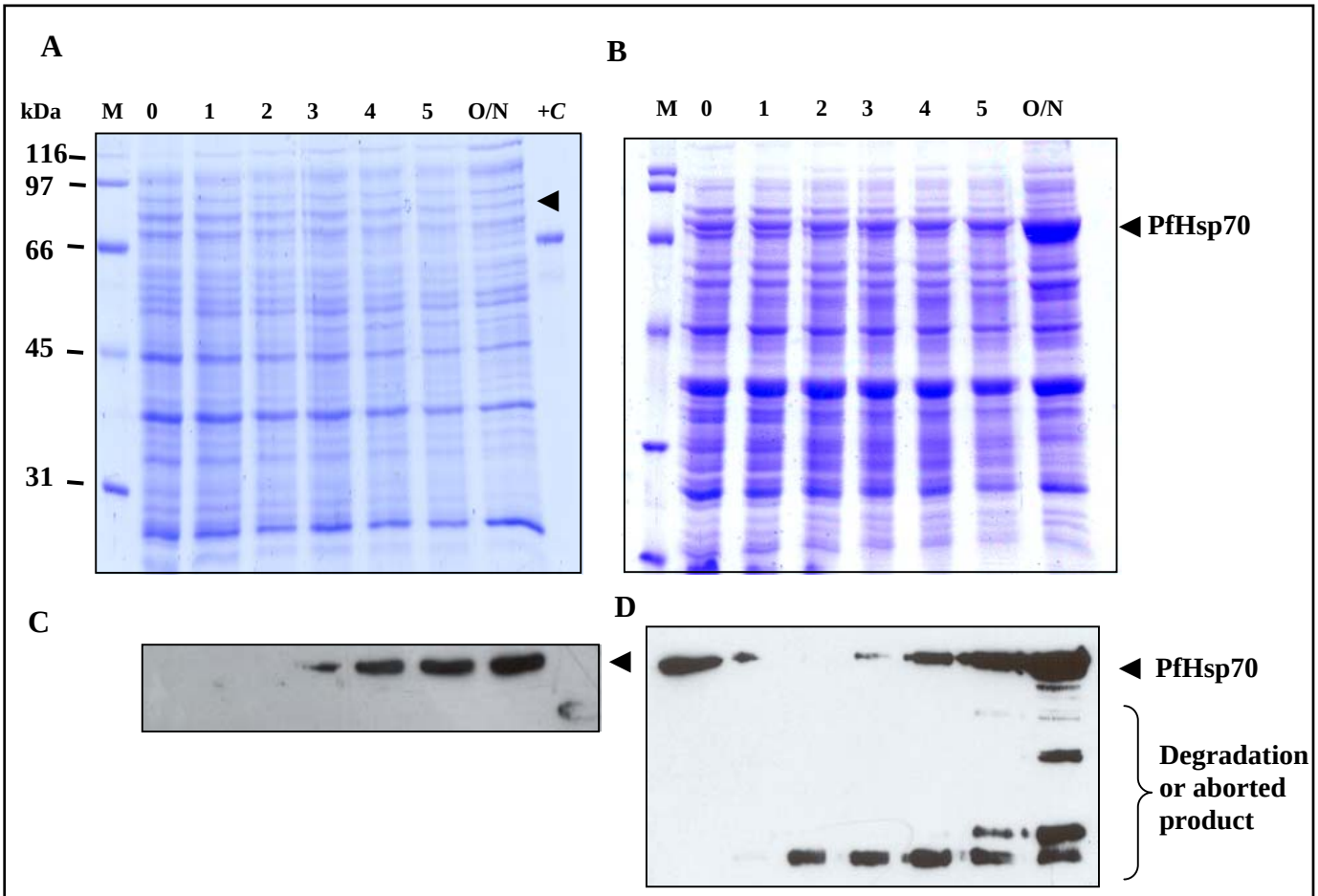


Figure 3.3: Comparison of Heterologous Expression of PfHsp70 in pRSETb and pQE30 vectors at 37˚C in *E.coli* BL21 (DE3) (pLySs) A and B: SDS-PAGE gels of heterologous production of PfHsp70 in *E. coli* BL21 (DE3) (pLys S) [pRSETb/PfHsp70] and *E.coli* BL21 (DE3) (pLys S) [pQE30/PfHsp70] respectively. Induction was done using 1 mM IPTG at 37 ºC. M (molecular mas markers), lane 0: non induced protein, lanes 1 to 5: Samples of induced proteins using IPTG taken hourly, lane O/N: Overnight sample, lane +C positive control $(His)_6$-tagged mST1. Levels of expression of PfHsp70 in *E. coli* Bl21 (DE3) (pLys S) were much higher using pQE30 expression vector compared to pRSETb expression vector. C and D: Detection of $(His)_6$-PfHsp70 using chemiluminescence–based Western analysis. Detected multiple bands in D may be products of translation stalling or degradation of the protein.

3.3.3 EFFECT OF RIG PLASMID

Baca and Hol, (2000) engineered a plasmid that encodes tRNAs for the three codons for Arg, Ile, and Gly, which are rarely used in *E. coli*. This plasmid is therefore referred to as RIG (Arginine, Isoleucine, and Glycine). The RIG plasmid was therefore transformed into the *E. coli* construct BL21 (DE3) (pLysS) [pQE30 / PfHsp70]), but overexpression was lost perhaps as a result of three plasmids within the cell. When pQE30/PfHsp70 vector together with RIG plasmid were transformed into *E.coli* XL1 Blue, overproduction was restored (Figure 3.4B). Chemiluminescence–based Western analysis revealed that full-length protein was

produced as the major protein as a result of the rare codons being successfully recognized (Figure 3.4C).

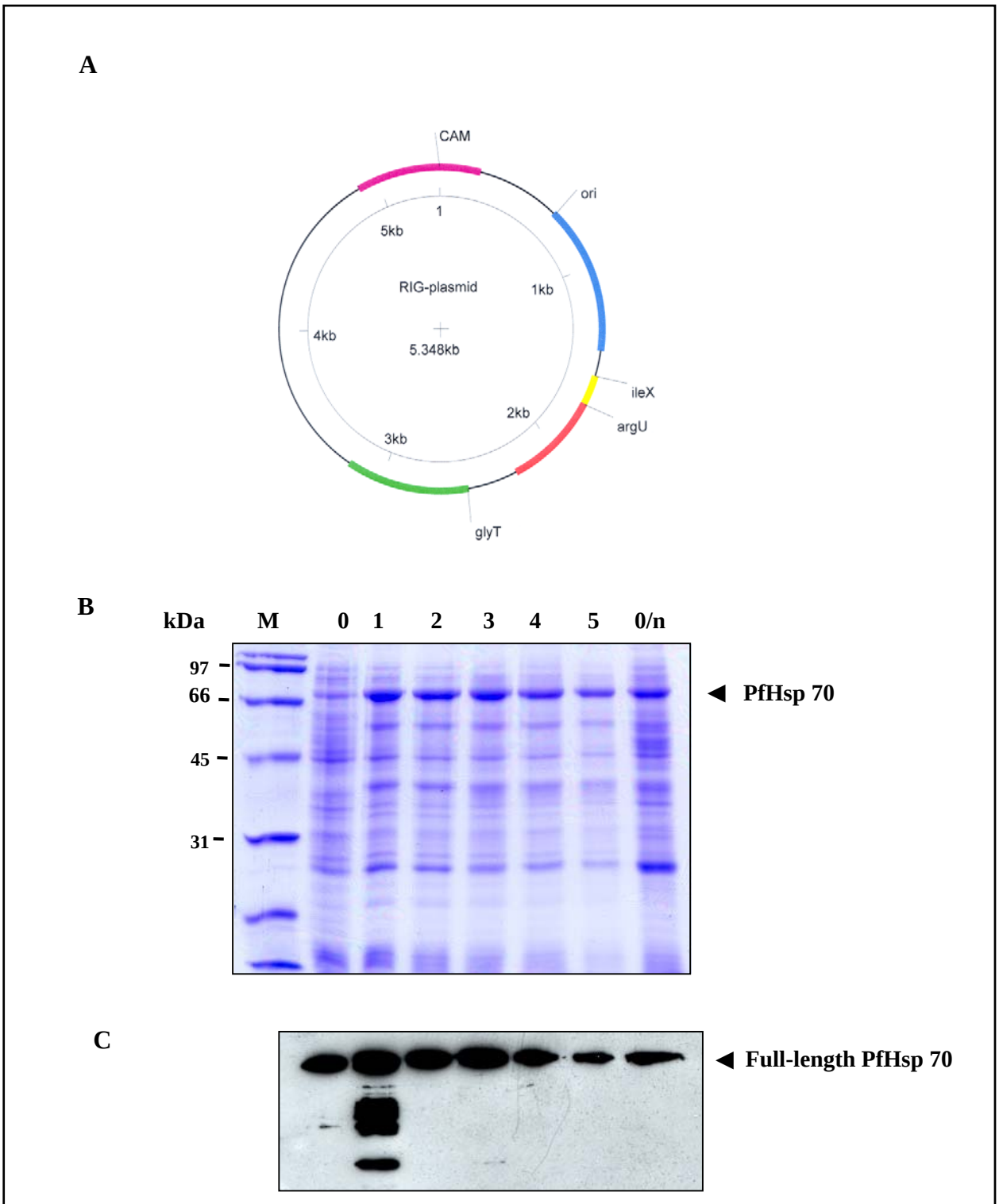


Figure 3.4: Heterologous production of PfHsp70 in *E.coli* XL1 Blue [pQE30/PfHsp70] (RIG)
(A) Plasmid map of the RIG plasmid showing the gene that encode for t-RNAs (Arg, Ile, Gly) which are rare codons in *E.coli*. (B and C) RIG plasmid encodes for rare t-RNAs as a result after 1 hour induction with IPTG there were traces of degradation products. But from 2 hour to 16 hours full length protein was expressed prossibly as a result of t-RNAs for rare codons being utilized. M: Molecular mass marker.

3.3.4 SOLUBILITY STUDIES OF RECOMBINANT $(His)_6$-PfHsp70

Protein purification can be carried out under either native or denaturing conditions. Solubility studies reveal the best route to use in order to achieve purification by determining whether the protein of interest is soluble or insoluble. It was revealed that PfHsp70 was largely present in the insoluble faction (Figure 3.5). This finding resulted in carrying out Ni- Chelating affinity purification under denaturing conditions using 8 M urea.

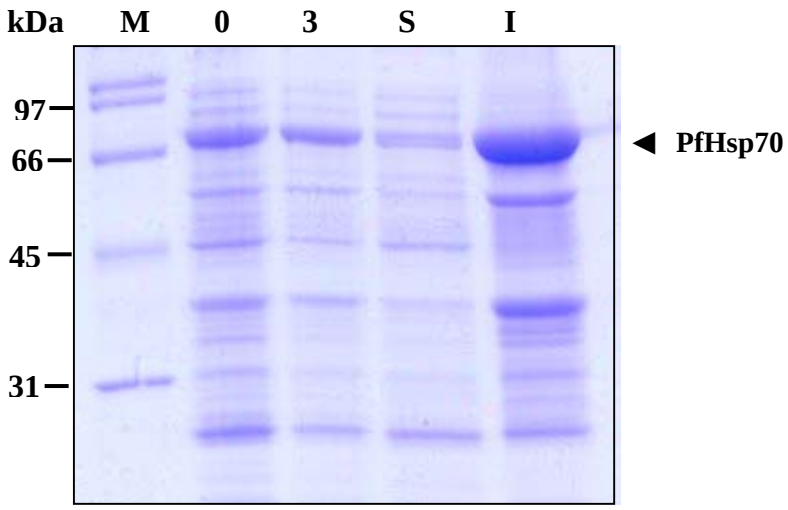


Figure 3.5: Determination of protein solubility in *E.coli* (XL1 Blue)

Solubility studies were carried out in *E.coli* XL1 Blue [pQE30/PfHsp70][RIG] using native lysis buffer. Results show that the protein (PfHsp70) is an insoluble protein (lane: I). Lane M: Molecular mass markers, lane 0: Non-induced protein, lane 3: Induction after 3 hours, lane S: Soluble proteins, lane I: Insoluble proteins.

3.3.5 PURIFICATION OF RECOMBINANT $(His)_6$-PfHsp70

Ni-Chelating affinity purification of $(His)_6$-tag PfHsp70 was carried out under denaturing conditions due to the fact that the protein was insoluble. Urea (8 M) was used to denature all proteins, allowing for complete exposure of the His-tag. In order to biochemically characterise (for instance carrying out ATPase assays) the protein was required in its functional state. In overcoming these conditions, lysis was carried out under denaturing conditions whereas washes and elutions were achieved under native conditions (i.e without urea). This enabled the denaturants to be diluted out, thereby allowing the protein to regain its structural and functional abilities. Lanes

labelled N and I show over-production of the protein. In the flow through (lane: F) a considerable amount of protein was lost due to the Ni-sepharose resins used for $(His)_6$-tag PfHsp70 binding was saturated with protein, therefore excess protein was lost. Washes 1 to 3 were carried out in order to remove any unspecific bound protein to the Ni-beads. From 1 litre of culture 1.93 g of PfHsp70 was obtained (lanes: E1). The concentration of protein purified ranged between 16 µM to 27.6 µM. Contaminants were noted below 6xHis-tag PfHsp70 band, this could be due smaller molecular mass fragments which were not completely removed during washes.

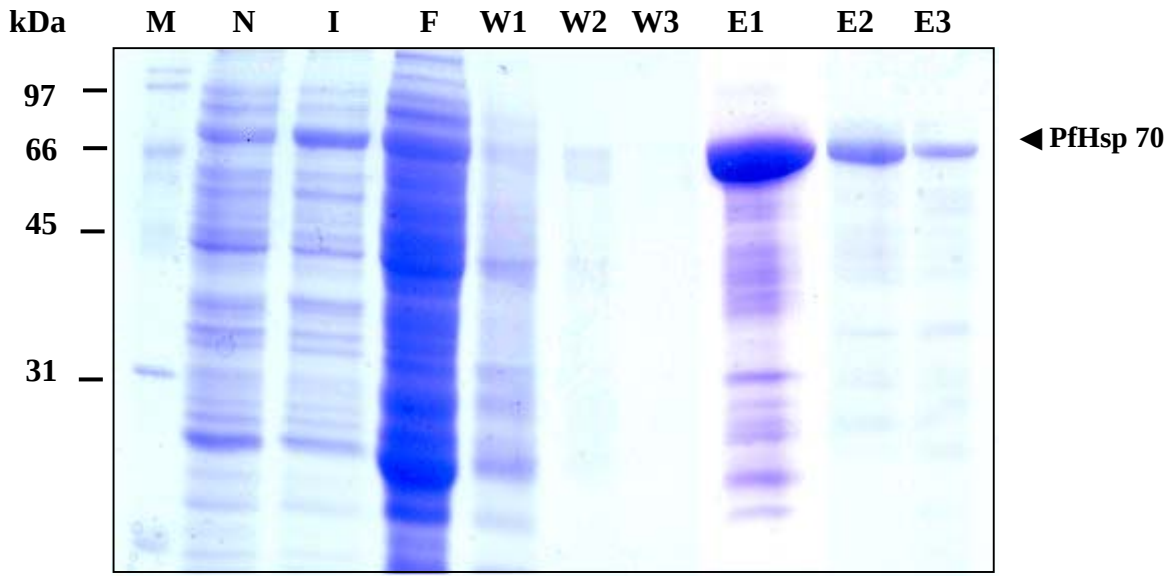


Figure 3.6: Batch Purification of $(His)_6$-tag PfHsp70 form *E.coli* XL1 Blue [PQE30 /Pfhsp70] [RIG].

Lane M: Molecular mass Markers, Lane 0: Noninduced whole cells, Lane 2: Induction of Whole cells after 2 hours, Lane F: Flow through, Lanes W1-3: Washes 1-3 (gradient washes using 50, 100 and 150 mM imidazole respectively), Lanes E1-3: Elutions 1-3 using 1 M imidazole,

3.3.6 SIZE EXCLUSION SE-HPLC ANALYSIS

The molecular mass of PfHsp70 was determined by gel filtration chromatography using SE-HPLC. Two peaks were observed; one implied a monomeric state and the other a dimeric or Trimeric (Figure 3.7A). Peak fractions were collected and analysed on a 12 % SDS PAGE gel. The bands detected on SDS-PAGE gel corresponded to the monomer and dimer forms of PfHsp70 (Figure 3.7B). An estimated molecular weight of 73.5 kDa and 199.5 kDa were obtained for the monomer and dimer, respectively. The 199.5 kDa was too large to be a dimer but also too small to be a trimer. It therefore formed an intermediate complex between a dimer and trimer. It was noted that PfHsp70 exists mainly in the monomer form as this is indicated by the sharp peak produced predominately where 73.5 kDa was calculated. Blue dextran was used in determining the void volume of the column and as a reference point.

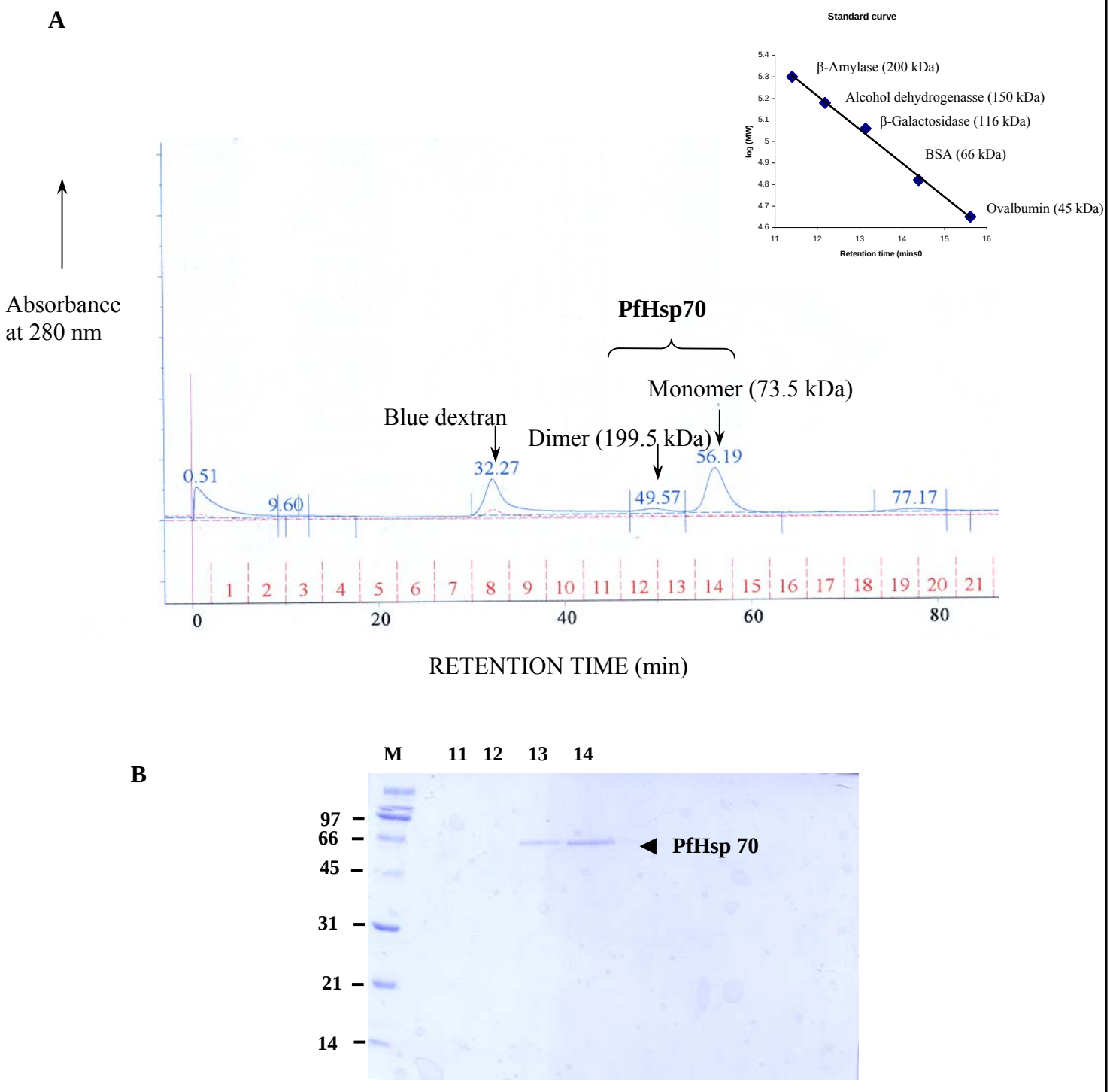


Figure 3.7: SE-HPLC analysis of PfHsp70

(A)PfHsp70 profile showing monomer and dimer, a concentration of 9.35 µM of PfHsp70 and 2 mg/ml of blue dextran was injected in the SEC-HPLC at a flow rate of 0.5 ml/min. The molecular weight of both monomer and dimer were found to be 73.5 and 199.5 kDa respectively. Insert shows standard curve for the molecular mass standards. (B) SDS-PAGE gel confirming PfHsp70. Lanes: 11, 12, 13 and 14 are fractions collected from the column M; Molecular mass markers.

3.3.7 NATIVE GEL ANAYLSIS OF COMPLEX FORMATION

When PfHsp70 was incubated with RCMLA, a protein in its native conformation resembling an unfolded protein (Palleros, *et al*., 1991), a higher molecular mass band than of PfHsp70 was detected on a 10% native PAGE gel (Figure 3.8, lanes 3 and 5). This band may either be a dimer form of Pfhsp70 or a complex formation between Pfhsp70 and RCMLA. Western analysis using Anti-Hsp70 and Anti-RCMLA antibodies would clearly reveal the identity of the higher molecular band.

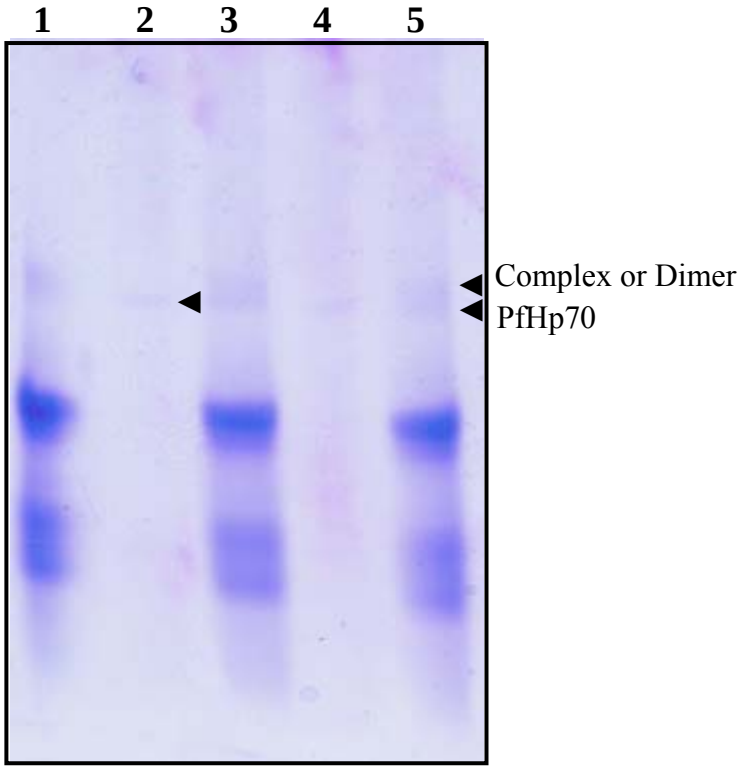


Figure 3.8: Confirmation of complex formation between PfHsp70 and RCMLA.

RCMLA (0.25 µg/µl) was incubated with PfHsp70 (0.25 µg/µl) at 37 ºC in the presence and absence of ATP. Samples were immediately loaded onto a 10% native PAGE gel. Lane 1: RCMLA only, lane 2: PfHsp70 only, lane 3: PfHsp70 and RCMLA, lane 4: PfHsp70 in the presence of ATP, lane 5: PfHsp70 and RCMLA in the presence of ATP.

3.3.8 SE-HPLC ANALYSIS OF COMPLEX FORMATION

Detection of PfHsp70 was problematic; a very broad major peak was detected for the monomer (Figure 3.9A) unlike those detected in figure 3.7A. Two peaks were detected for RCMLA; a sharp peak at 15 kDa and broad peak 22.3 kDa (Figure 3.9B). A molecular mass of 15 kDa was determined which is within the known range of about 14 kDa for RCMLA, however the 22.3 kDa form is too small to be a dimer. When PfHsp70 was incubated with RCMLA at 37ºC for 30 minutes and immediately loaded onto the SE-HPLC, The major peak for RCMLA shifted to lower retention time suggesting a higher molecular mass (Figure 3.9C). This shift may indicate a conformational change in RCMLA, with a molecular mass of 22.3 kDa. PfHsp70 was detected at a molecular mass of 111.4 kDa; this may be (i) the result of a complex formation with RCMLA, (ii) a conformational change in PfHsp70 to a more extended form or (iii) a dimmer of PfHsp70 with a compact conformation. Due to the small amount of protein loaded onto the SE-HPLC it was very difficult to analysis the fractions collected on a native PAGE gel to confirm the conformational change detected on the SE-HPLC. These results provide evidence that PfHsp70 may be able to interact with unfolded protein (as represented by RCMLA) and therefore may have chaperone like activity. However further evidence such as further binding assays and folding/unfolding studies are needed.

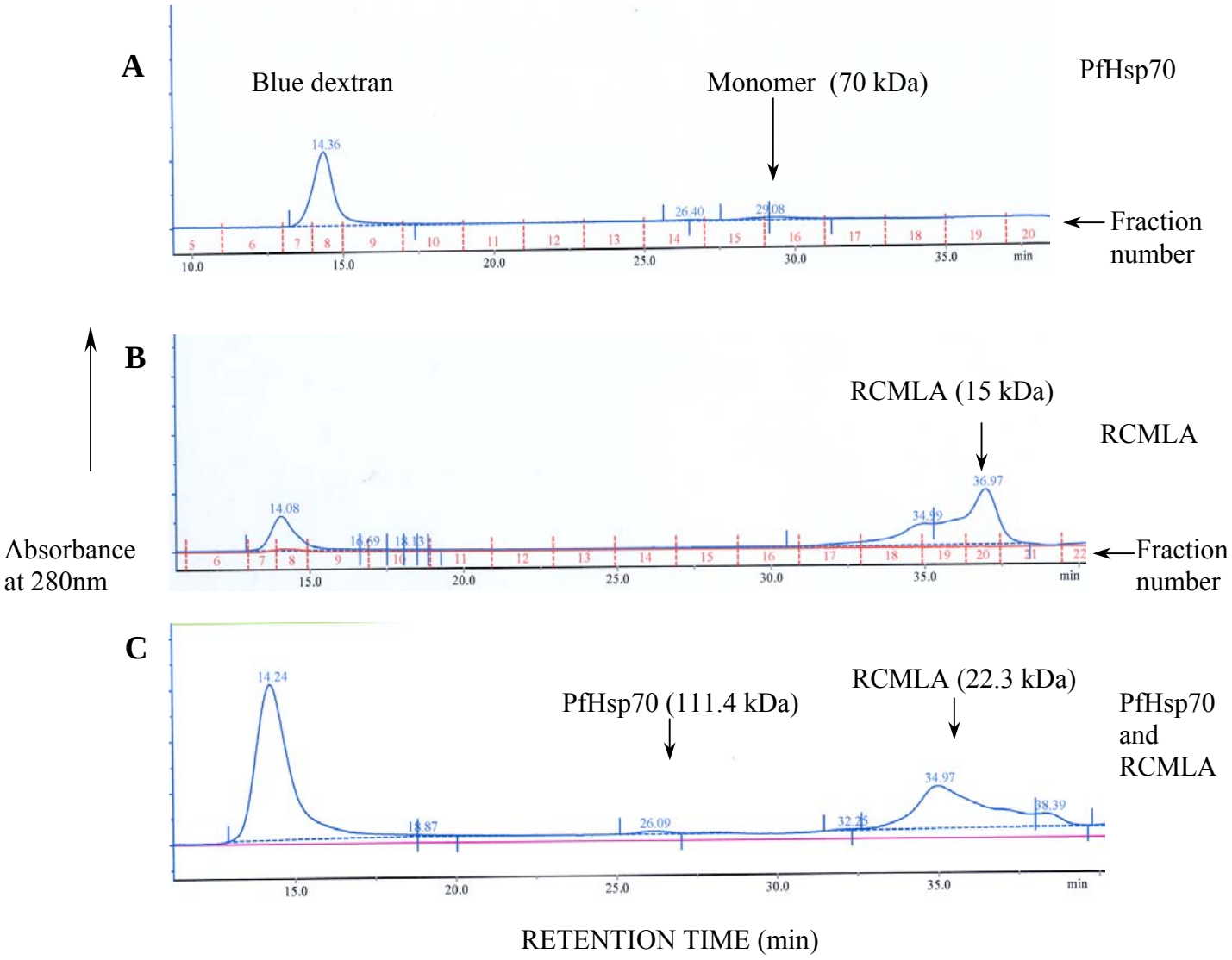


Figure 3.9: Binding of RCMLA to PfHsp70

Binding of RCMLA (40 µM) to PfHsp70 (18 µM) was achieved by incubating the protein at 37 ºC for 30 min. (A) SE-HPLC analysis of PfHsp70 alone, (B) RCMLA alone (C) PfHsp70 and RCMLA showing conformational change of RCMLA. Blue line represents Absorbance at 280 nm and Red line represents absorbance at 215 nm.

3.4 CONCLUSION

Over-production of PfHsp70 was improved with the use of pQE30 vector, this was achieved by inserting the start codon of PfHsp70 only 2 amino acids down stream of the 6xHis-tag. Multiple bands were detected on Chemiluminescence–based Western analysis revealed multiple bands, which may be the result of aborted or degradation products. Baca and Hol (2000) engineered a plasmid that encodes for tRNAs for Arg, Ile and Gly, which is referred to as the RIG plasmid. When the RIG plasmid was used full-length PfHsp70 was produced, probably as a result of the tRNAs recognizing the rare codons.

Purification of Pfhsp70 was achieved under denaturing conditions using 8 M urea. Refolding of the protein was achieved using native washes and elutions. This was done in order to further characterize the protein.

Gel filtration chromatography carried out on PfHsp70 revealed the protein to be more dominant in its monomeric form, however a larger molecular mass was observed with a broader peak. The higher molecular mass peak may be the result of a compact dimeric from. The estimated molecular mass of Pfhsp70 was found to be 73.5 kDa in its monomeric form and 199.5 kDa in the proposed compact dimeric form.

RCMLA binding studies revealed a possible complex formation with PfHsp70, this was analysed on a native PAGE gel. The complex was of a higher molecular mass than that of PfHsp70. This complex may either be a dimer form of PfHsp70. Further analysis using anti-Hsp70 and anti-RCMLA anti-bodies would determine the components of the higher molecular bands/complex.

Two peaks where observed for RCMLA on a SE-HPLC. The peaks were of molecular masses 15 kDa and 22.3 kDa. In the presence of PfHsp70 there was a conformational change in RCMLA, which resulted in a shift towards the 22.3 kDa, form. This conformational change may indicate chaperone activity by PfHsp70. Further evidence such as binding assays and folding/unfolding studies are required to support the predication.

CHAPTER 5

OVERALL DISCUSSION AND CONCLUSION

5.1 OVERALL DISCUSSION AND CONCLUSION

5.1.1 OVER PRODUCTION AND PURIFICATION

Expression of *Plasmodium* genes in *E. coli* has proven to be difficult, this is due to the high A-T rich genome of about 80 % (Baca and Hol, 2000), whereas *E. coli's* genome is more biased towards G-C. This causes a major problem in expressing certain *Plasmodium* genes in *E. coli* due to rare codon usage. There are three main codons that are rarely used in *E.coli*; these are Arg (AGA/AGG), Ile (AUA) and Gly (GGA). The original plasmid (pRSETb/PfHsp70) obtained was problematic; expression was extremely low but a considerable amount of protein was purified using Ni-chelating affinity column. DNA sequencing carried out near the start codon of PfHsp70 revealed a hydrophobic linker region between the start codon and the 6xHis-tag. We therefore inserted the PfHsp70 coding region into the pQE30 expression vector, which encodes the N-terminal 6xHis-tag. DNA sequencing analysis at the start of the coding region revealed that only two amino acids were encoded down stream of the 6xHis-tag codons. Expression in *E. coli* was improved, but multiple bands were detected on chemiluminescence–based Western analysis. These multiple bands may be the result of aborted or degradation products. There are two ways in which full-length protein may be produced: (i) by changing the rare codons of the *Plasmodium* gene to those recognised in *E. coli*, or (ii) To engineer an *E. coli* strain with a plasmid that produces tRNAs that recognises the rare codons. In our studies we opted for engineering an *E. coli* strain with the plasmid that produces the tRNAs since previous studies carried out in *Plasmodium* genes successfully used this method (Baca and Hol, 2000). A RIG plasmid that encodes for tRNAs for Arg, Ile and Gly was engineered by Baca and Hol (2000). When the RIG plasmid was used full-length PfHsp70 was produced, probably as a result of the tRNAs recognizing the rare codons. Purification was carried out under denaturing conditions using 8 M urea, since solubility studies revealed that the protein was insoluble. The protein was refolded using native washes and elutions. This was done in order to further characterize the protein.

5.1.2 ATP HYDROLYSIS OF PfHsp70

Basal ATP hydrolysis of PfHsp70 was found to be 4 times and 2 times lower than that of Hsc70 and Hsp70 respectively. This low activity may result from the denaturing conditions used during purification; a majority of the protein may not have fully refolded during native washes and elutions. RCMLA was found to stimulate PfHsp70 by 11 fold whereas Tcj2 and RCMLA stimulated the ATPase activity by 16-fold. These results may indicate that PfHsp70 may be involved in chaperone activity. It was observed that increasing the concentration Tcj2 showed a decrease in activity and, no activity was obtained with 1mM of Tcj2, this was observed in the presence and absence of RCMLA. These results may indicate that excess Tcj2 may block the binding site of ATP/ADP. Tcj2 was used as a co-chaperone to PfHsp70 in order to determine the stimulation by co-chaperones on the ATPase activity. Very little work has been carried out on the DnaJ proteins of *Plasmodium falciparum* (Pfj 1-4). This work would have aided in the demonstration of the chaperone activity and revealed to what extent Pfjs stimulates the ATPase activity of Pfhsp70.

5.1.3MECHANISTIC STUDIES OF RATE LIMITING STEP

In determining the rate-limiting steps in ATPase activity, ATP hydrolysis kinetics and the ADP release kinetics experiments were carried out. The calculated k_{cat} (0.003 min^{-1}) of ATP hydrolysis had a lower value than that of ADP release (k_{cat} 0.806 min^{-1}). These results revealed that the rate of ADP release from the ATP/ADP cleft of PfHsp70 was fast compared to the rate of ATP hydrolysis. This may indicate that ATP hydrolysis maybe the rate-limiting step during ATPase activity. Work carried out by Jonathan *et al*, (1999) also showed that ATP hydrolysis was the rate-limiting step in *E. coli*. In the presence of cochaperone ATP hydrolysis is increased suggesting that cochaperones may be required to increase the chaperone activity of PfHsp70.

5.1.4 MOLECULAR MASS DETERMINATION

Gel filtration chromatography carried out on PfHsp70 revealed the protein to be more dominant in its monomer form, however a larger molecular mass was observed with a broader peak. The higher molecular mass peak may be the result of a compact dimer from. Nadia *et al,* 1996 have shown that temperature shift from 15-37 °C on Hsc70 tends to increase the mononer form, from 70 to 90 %. Work also carried out by Palleros *et al,* (1991) showed Hsp73 to be more dominate in its monomer form. The estimated molecular mass of Pfhsp70 was found to be 73.5 kDa in its monomer form and 199.5 kDa in the proposed compact dimer form.

5.1.5 SE-HPLC ANALYSIS OF COMPLEX FORMATION

Binding studies with RCMLA analysed on a native PAGE gel revealed the possibility of a complex formation between PfHsp70 and RCMLA, or dimer form of PfHsp70 of higher molecular mass than that of PfHsp70. Further evidence using anti-Hsp70 and anti-RCMLA anti-bodies would determine the componants of the higher molecular bands/complex. SE-HPLC analysis revealed two peaks for RCMLA; a sharp and a broad peaks of molecular masses 15 kDa and 22.3 kDa respectively. RCMLA is known to have a molecular mass of about 14 kDa, which corresponds to the sharp 15 kDa peak. A conformational change in RCMLA could account for the shift towards the 22.3 kDa, form in the presence of PfHsp70. This conformational change may indicate chaperone activity by PfHsp70. Further evidence such as binding assays and folding/unfolding studies are required.

5.1.6 FUTURE WORKS

This study has been the first to show that PfHsp70 may act as a molecular chaperone, however extensive work is required to provide more evidence to support these predictions. Future work is required on determining the ATP hydrolysis of PfHsp70 in the presence of cochaperone such as *Plasmodium falciparum* DnaJs (Pfj 1-4). *In vivo* complementation studies in prokaryotic and eukaryotic, Hsp70/DnaK and Hsp40/DnaJ deficient cells in the presence of Pfjs may provide further evidence of chaperone activity by PfHsp70. Domain swapping of Pfhsp70 with those of DnaK

may reveal functionally important domains within PfHsp70. Our bioinformatic studies revealed a predicted structure of the ATPase domain of PfHsp70, however the three dimensional structure may be determined by x-ray crystallography.

APPENDIX A

SUPPLEMENTARY RESULTS

A1: AMPLIFICATION OF PfHsp70 AND INSERTION INTO pGEM-T VECTOR.

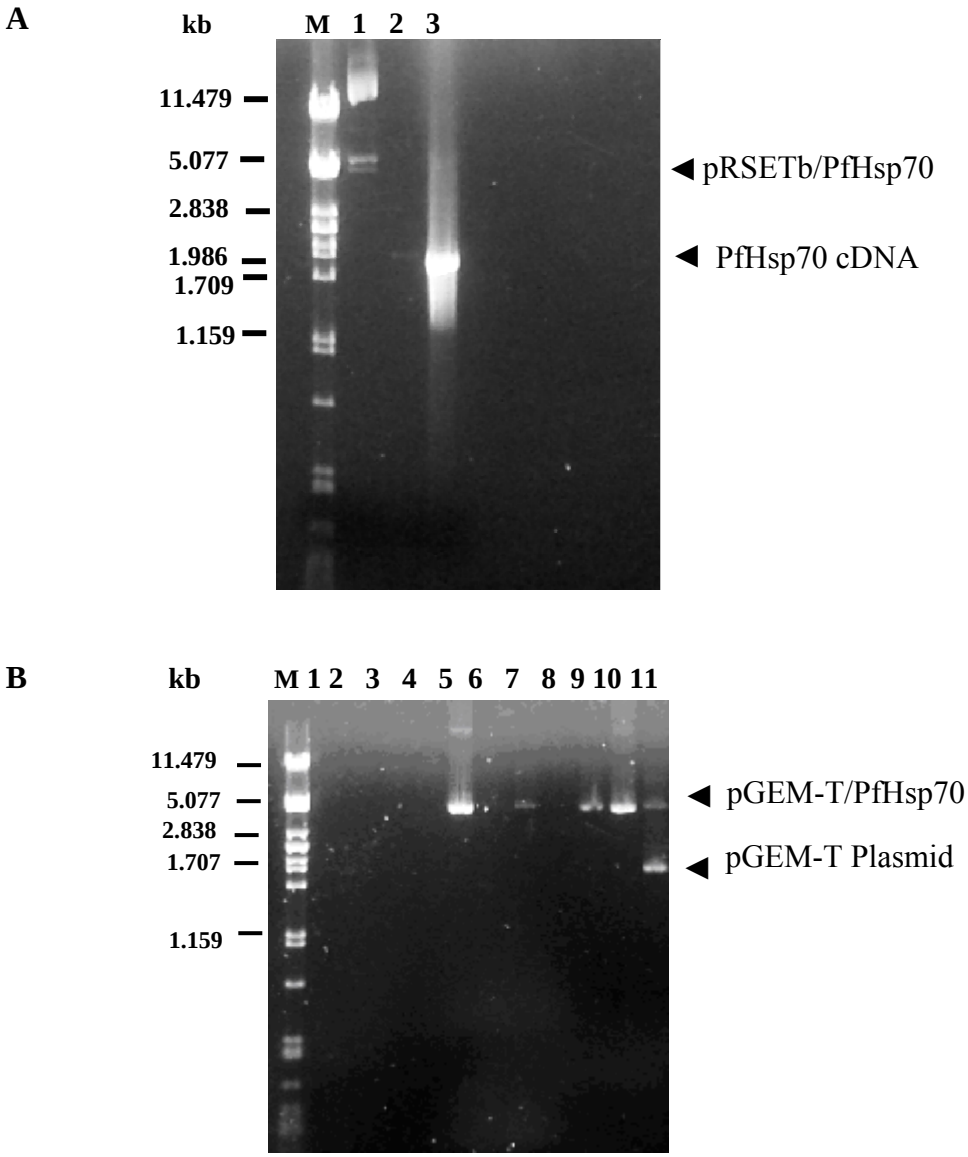
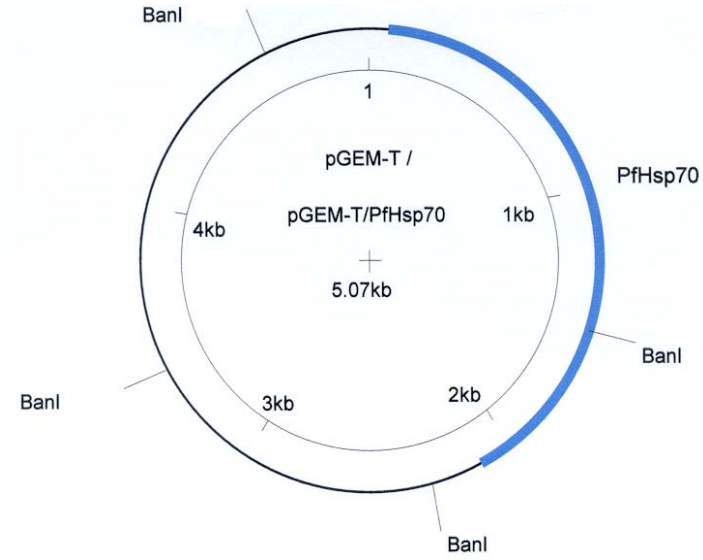


Figure A1: PCR product of PfHsp70 from pRSETb/PfHsp70 vector and plasmid isolation of pGEM-T/PfHsp70

Agarose gels. (A) PCR product of PfHsp70 cDNA. M; Molecular mass marker. Lane 1; pRSETb/Pfhsp70 plasmid. Lane 2; PfHsp70 cDNA cut out from pRSETb/PfHsp70 plasmid using *Bgl* II and *Hind* III enzymes. Lane 3; PCR product of PfHsp70 cDNA.(B) Isolation of pGEM-T/PfHsp70 from *E.coli* BL21 (DE3)(pLySs) using conventional small-scale plasmid DNA preparation. M; Molecular mass marker, Lanes1-10: pGEM-T/PfHsp70 plasmid. Lane 11: pGEM-T plasmids without inserts (PfHsp70).

A2a: CONFIRMATION OF pGEM-T/PfHsp70 CLONE USING BanI ENZYME

A



B

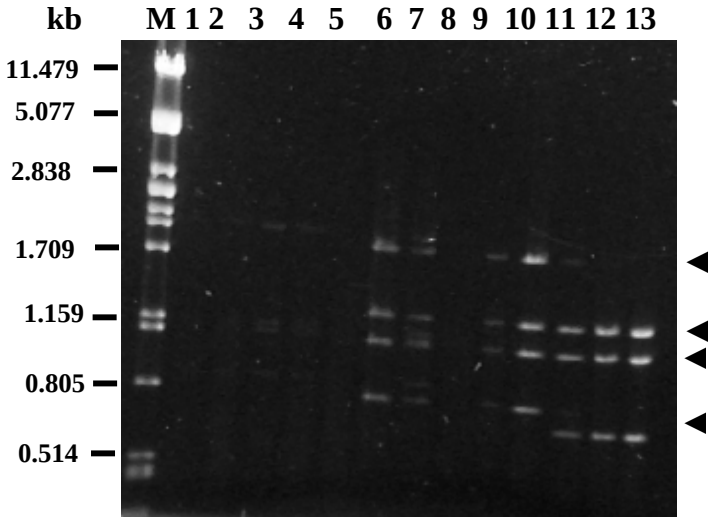
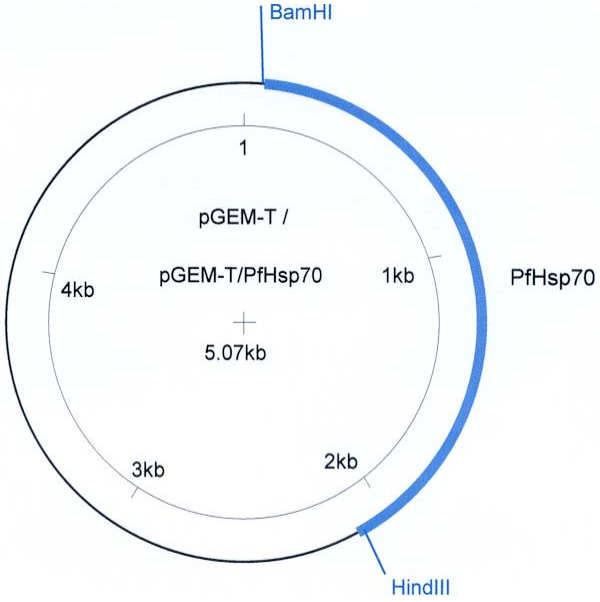


Figure A2a : Confirmation of pGEM-T/PfHsp70 constructs.

(A)Plasmid map pGEM-T/PfHsp70 showing restriction sites of BanI .(B) Agarose gels of pGEM-T/PfHsp70 digested with *Ban*I M; Molecular mass marker (λ DNA cut with Pst 1). Lanes 1-10: pGEM-T vectors containing PfHsp70 insert. Lanes 11-13: pGEM-T vector without inserts. When digested with *Ban*I restriction enzyme four fragments are produced (arrows indicate expected bands), 3 cut are within pGEM-T and only 1 within the coding region of PfHsp70.

A2b: CONFIRMATION OF pGEM-T/PfHsp70 CLONE USING BamHI AND HindIII ENZYMES

A



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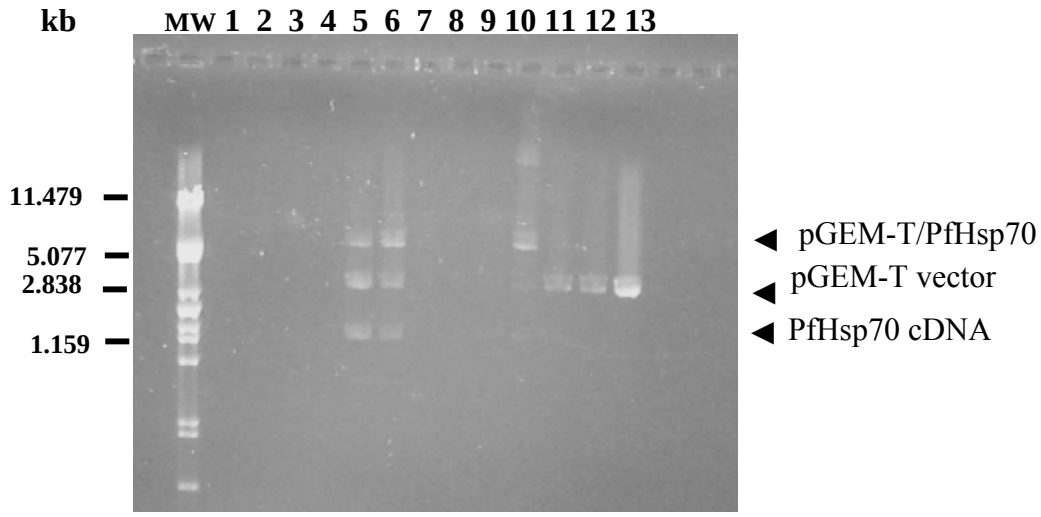


Figure A2b : Confirmation of pGEM-T/PfHsp70 clone.

(A) Plasmid map of pGEM-T/PfHsp70 showing restriction sites of *BamH*I and *Hind*III. (B)Agarose gels of pGEM-T/PfHsp70. double digested with *BamH*I and *Hind* III. M; Molecular mass marker (λ DNA cut with Pst 1) Lanes 5-6; shows partial digestion of pGEM-T/PfHsp70, pGEM-T/PfHsp70 vector upper band, pGEM-T vector middle band, and PfHsp70 cDNA lower band. Lanes 11-13; pGEM-T vectors without inserts.*BamH*I and *Hind*III release PfHsp70 cDNA.

A3: CONFIRMATION OF RIG PLASMID USING Sac1 AND Nhe1 ENZYMES

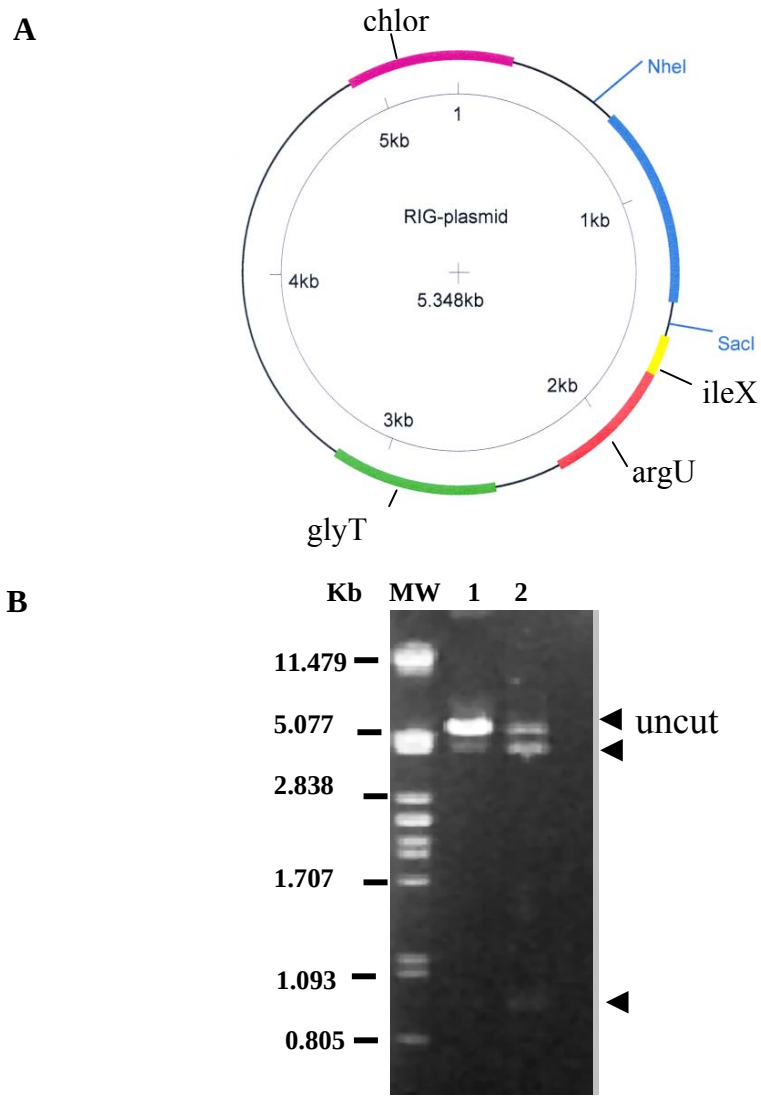


Figure A3:confirmation of RIG plasmid

(A) Plasmid map of RIG plasmid showing *Nhe*I and *Sac*I restriction sites.(B) Agarose gel of RIG plasmid .Lane M; Molecular mass marker (λ DNA cut with Pst 1), lane 1: Uncut plasmid(5,348 Kb), lane 2: plasmid double digested with *Nhe*I and *Sac*I,producing 2 fragments of sizes 4391 and 957 bases,and an uncut due to partial digestion ilex: Isoleucine coding region, argU: arginine coding region, glyT: glycine coding region, chlor: Chloramphenicol coding region.

A4 :PROTEIN DETERMINATION USING THE BRADFORD's ASSAY

Table B1: Protein Determination by Bradford's Assay

Sample	Vol of mg/ml BSA stock (µl)	Vol of 0.15 M NaCl	Concentration (µg/ml)
Blank	0	100	0
1	2	98	20
2	4	96	40
3	6	94	60
4	8	92	80
5	10	90	100

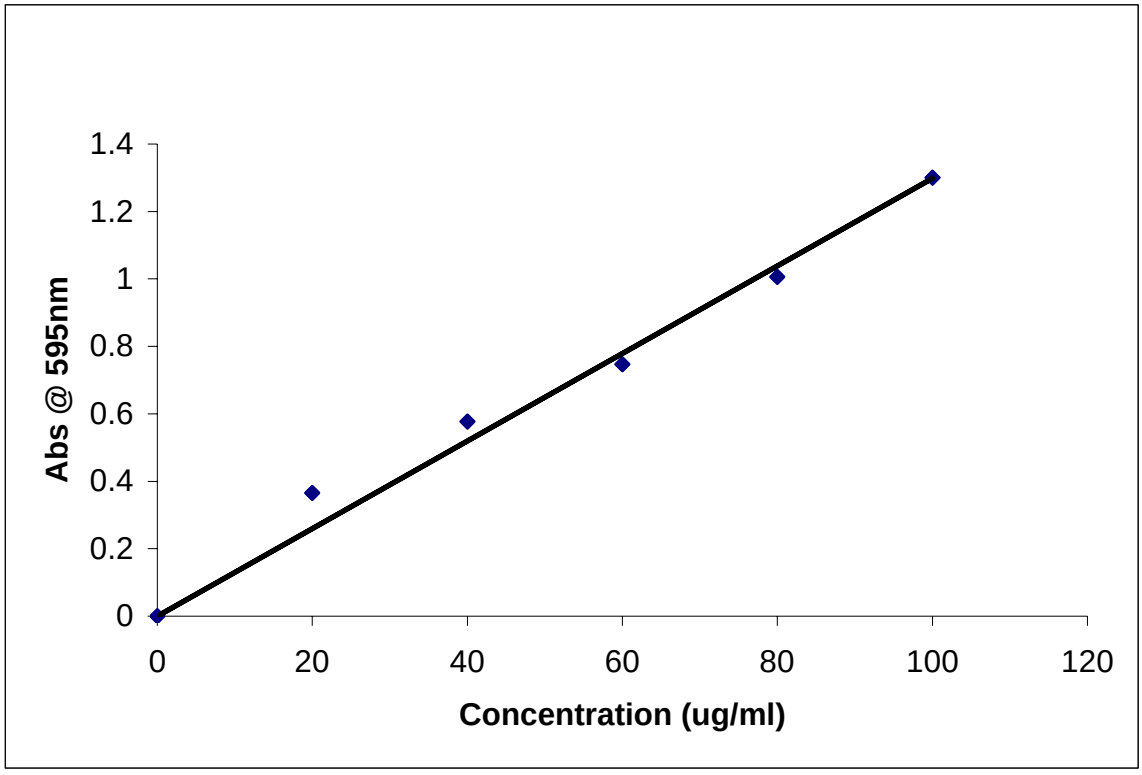


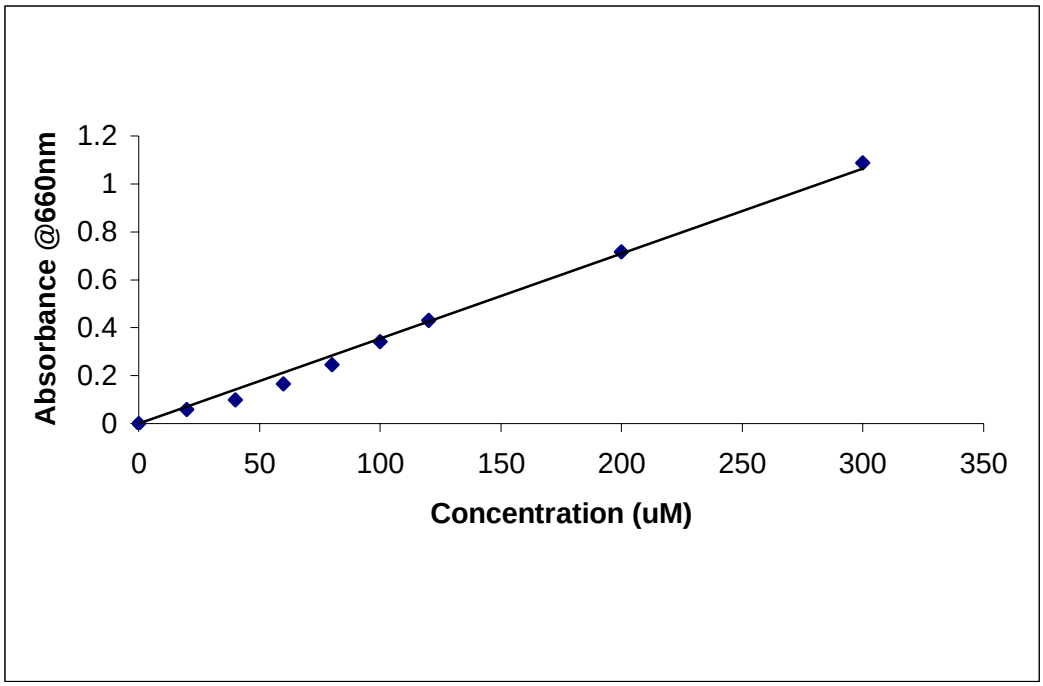
Figure A4: Protein Standard Curve

Bradford's reagent was used to determine the concentration of PfHsp70. The standard curve had an R^2 value of **0.9845** and the equation used was y=0.0122x.

A5: PHOSPHATE STANDARD CURVE

Table A2: Phosphate Standard curve

Solution	1	2	3	4	5	6	7	8	9
Na_2HPO_4 (µl) [Conc(µM)]	- 0	2 20	4 40	6 60	8 80	10 100	12 120	20 200	30 300
H_2O (µl)	100	98	96	94	92	90	88	80	70
1.25% Ammonium molybdate (µl)	50	50	50	50	50	50	50	50	50
9% Ascorbic acid (µl)	50	50	50	50	50	50	50	50	50



FigA5: Phosphate standard curve

The standard curve was used for the determination of ATP hydrolysis enzyme activity by measuring the phosphate (Pi) concentration produced. R^2 value of **0.9938** and the equation used was y=0.0035x

A6: ATPase ASSAY

Table A3; ATPase Assay

Solution	
100 mM HEPES	40 µl
1 M KCl	40 µl
100 mM $MgCl_2$	8 µl
10 mM Ditheothreitol (DTT)	20 µl
Ultra pure H_20 (make up to 200µl)	92 µl
0.4 µM Protein (appropriate amount)	X µl
Ultra pure H_20 (make up to 400µl-Z)	400-Z µl
Incubate for 5 mins @ 37°C	
4 mM ATP (Start reaction)	Z µl

NOTE: The above is a reaction mixture from which samples (50 µl) are collected over time interval. The below reagents are added to the sample and assayed for Pi produced, the total reaction volume is 200 µl.

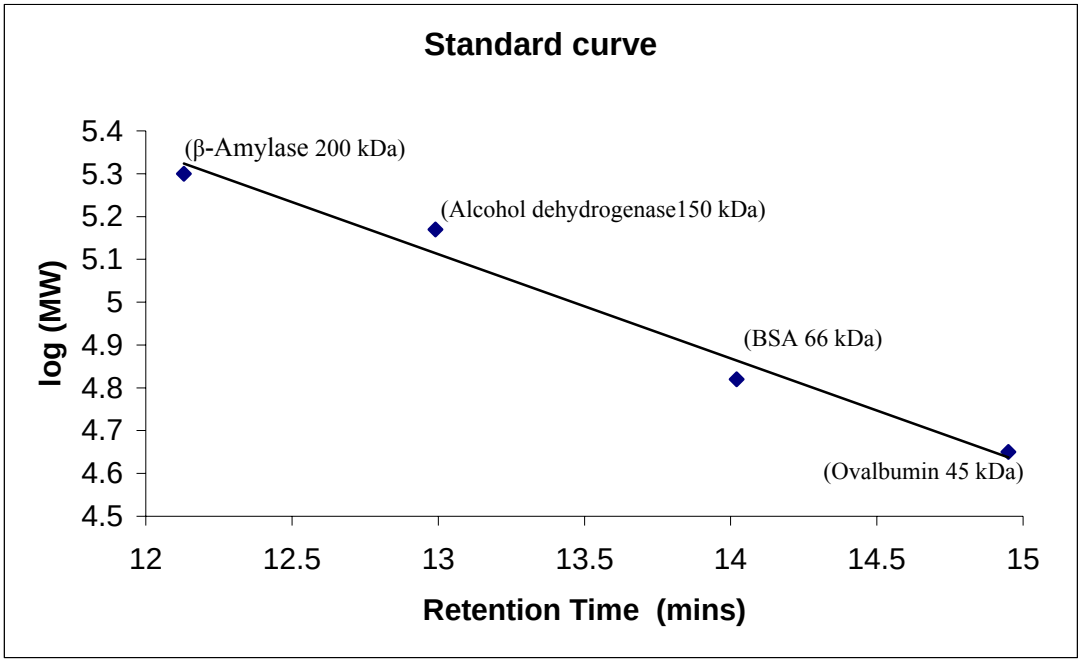
∗10% SDS (Stop reaction)	50 µl
†1.25% Ammonium molybdate	50 µl
†9% Ascorbic acid	50 µl

∗ SDS stops reaction by denaturing the protein and **NO CRYSTALS** are formed preventing scattering of light during absorbance reading.

† 1.25% Ammonium molybdate and 9% Ascorbic acid assay for inorganic phosphate (Pi) released during ATP hydrolysis.

Allow to stand at room temperature for 30 minutes and read absorbance at 660 nm.

A7: MOLECULAR MASS STANDARD CURVE FOR SE -HPLC



FigA7: Molecular weight standard curve

Molecular weight of PfHsp70 was determined using the log of molecular weights of known proteins. The R^2 value was **0.979** and the equation used was y= -0.2435x+8.2771.

REFERENCES

Ait-Aisse, S., Porcher,J., Arrigo, A., and Lambre, C. (2000) Activation of the hsp 70 promter by environment inorganic and organic chemicals. Relationship with cytotoxicity and lipophilicity. *Toxicology* .**145,** 147-157

Arrigo, A. (1998) Small proteins: chaperones that act as regulators of intracellular redox state and programmed cell death. *Biol.Chem.* **379,** 19-26

Baca, A. M ., and Hol, W. G. J. (2000) Ocercoming codon bais: A method for high-level overexpression of *Plasmodium* and other AT-rich parasite genes in *Escherichia coli. Int. J. Parasite.* **30,** 113-118

Bimston, D., Song.J., Winchester.D., Takayama.S., Reed.J.C., and Morimoto.R. (1998) BAG-1, a negative regulator of Hsp70 chaperone activity, uncouples nucleotide hydrolysis from substrate release .*EMBO.* **17 (23),** 6871-6878

Birren, B., Green, E.D., Klapholz, S., Myers, R.M., Riethman, H., and Roskams, J. (1999) In *Genome Analysis a laboratory Manual, Cloning System volume 3* pp. 1, 7-8, Cold spring Harbor Laboratory Press, USA

Biswas, S., and Sharama, Y. (1994) Enhanced expression of *P.falciparum* heat shock protein Pfhsp 70-1 at higher temperatures and parasites survival. *FEMS Microbiol Lett.* **124,** 425-9

Blatch, G.L., and Lässle, M. (1999) The tetratricopeptide repeat:a structural motif mediating protein-protein interactions.*BioEssays* .**21,**932-939

Bork, P., Sander, C., and Valencia, A. (1992) An ATPase domain common to prokaryotic cell cycle proteins, sugar kinases, actin, and hsp70 heat shock proteins. *Prc. Natl. Acad. Sci*.USA .**89**,7290-7294

Bradford,M.M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Analy Biochem.* **72,** 248-254

Bricker, A., Scheufler, C., von der Mülbe, F., Fleckenstein, B., Herrmann, C., Jung, G., Moarefi, I., and Hartl, F. (2002) Ligand Discrimination By TPR Domains .*J.Biol Chem.* **277(22),** 19265-19275

Chamberlain, L. H and Burgoyne, R.D (1997) Activation of the ATPase activity of heat-shock proteins Hsc70/Hsp70 by cysteine-string protein. *Biochem. J.* **322,** 853-858

Cheetham,M. E., and Caplan, A. J. (1998) Structure, function and evolution of DnaJ: conservation and adaptation of chaperone function. *Cell Stress & Chaperones*. **3** (1), 28-36

Chinea, G., Pardrsn,G., Hooft, R. W. W., Sander, C ., and Vriend,G (1996) The Use of PositionSpecific Rotamer in Model Building by Homology.*Proteins.***23,** 415-421

Combet, C., Jambon, M., Deleage, G., and Geourjon, C. (2002) Geno3D: automatic molecular modelling of protein. *Bioinformatic.* **18,** 213-214

Cuff, J. A., Birney, E., Clamp, M. E., and Barton, G. J (2000) ProtEST: Protein multiple sequence alignments from expressed sequence tags. *Bioinformatics.* **16,** 111-116

Flaherty, K.M., DeLuca-Flaherty, C.,and Mckay, D. B (1990) Three-dimensional structure of the ATPase fragment of a 70K heat-shock cognate prtein. *Nature* **346,** 623-628

Freeman, B. C., Myers, M.P., Schumacher, R., and Morimoto, R.I. (1995) Identification of a regulatory motif in Hsp7 that affects ATPase activity, substrate binding and interaction with HDJ-1. *EMBO.* **14,** 2281-2292

Gentz, R., Chen, C.H., and Rosen, C. A. (1989) Bioassay for trns-activation using purified human immunodeficiency virus tat-encoded protein: trans-activation requireds mRNA synthesis. *Proc.Natl.Acad.Sci*.**86,** 821-824

Hiromura, M., Yans, M., Mori, H., Masshiro, I., and Hiroshi, K. (1998) Intrinsic ATP-ADP exchange Activity is Novel function of the molecular chaperone Hsp 70. *J.Biol Chem.* **273,** 5435-5438

Hochuli, E., Dobeli, A., and Schacher, A. (1987) New metal chelating adsorbent selective for proteins and peptides containing neighbouring histidine residues. *J. chromatogr*. **411,** 177-184

Hyde, J. E. (2002) Mechanisms of resistance of *Plasmodium falciparum* to antimalarial drugs. *Micro. and Inf.* **4,** 165-174

Joshi, B., Biswas, S., and Sharama, Y. (1992) Effects of heat-shock on P.falciparum viability growth and expression of the heat-shock PfHSP 70-1 gene.*FEB Lett* .**12(1),** 91-94

Kamath-loeb, A.S.,Lu, C., Suh, W., Lonetto, M. A., and Gross, G.A (1995) Analysis of Three DnaK Mutant Proteins Suggests That Progression through the ATPase Cycle Requires Conformational Changes. *J.Biol Chem.* **270,** 30051-30059

Kane, J.F. (1995) Effect of rare codon clusters on high-level expression of heterologous proteins in *Escherhia coli*. Current opin in Biotech. **6,** 494-500

Kraulis,P.(1991). Journal of Applied crystallography. **24**, 946-950

Kumar, N., Zhao, Y., Graves, P., Folger, J.P., Maloy, L., and Zheng, H. (1990)

Human immune response directed against *P.falciparum* heat shock-repeated proteins. *Infect immune*. **58(5),** 1408-14

Kumar, N., and Zheng, H. (1992) Nucleotide sequence of a Plasmodium falciparum stress protein with similarity to mammalian 78Kd glucose-regulated protein. *Mol.Biochem Parasitology*. **56,** 3-356

Kumar, N., and Zheng, H. (1998) Evidence for epitope-specific thymus-independent response against a repeat sequence in a protein antigen. *Immunology*. **94,** 28-34

Laufen, T., Mayer, M.P., Beisel, C., Klostermeier, D., Mogk, A., Reinstein, J., and Bukau, B. (1999) Mechanism of regulation of Hsp70 chaperones by DnaJ cochaperones. *Prc. Natl. Acad. Sci.* USA.**96,** 5452-5457

Luders, J., Demand, J., Schonfolder, S., Frien, M., Zimmermann, R., and Hohfeld, J. (1998) Cofactor-induced Modulation of the functional specifity of the molecular chaperone Hsc 70. *Biol.Chem*. **379,** 1217-1226

Mayer, M., and Bukau, B. (1998) Hsp 70 chaperone systems: Diversity of cellular functions and mechanism of action. *Biol.Chem*. **379,** 261-268

Mayer, M. P., Schröder, H., Rüdiger, S., Paal, K., Laufen, T., and Bukau, B. (2000) Multiple mechanism of substrate binding determines chaperone activity of Hsp70. *Nature Stru Bio.***7 (7),** 586-593

Molinari, M., and Helenius, A. (2000) Chaperone selection during glycoprotein translation into the endoplasmic reticulum. S*cience*. **288,** 331-333

Morimoto, R. I., Kline, M. P., Bimston, D, N., and Cotto, J. J. (1997) The heat-shock response: regulation and function of heat-shock proteins and molecular chaperones. In Essays in Biochemistry volume 32 (Bowles, D. J., ed) pp.17-27, Portland and Press, London

Minami, Y., HöHfeld, J., Ohtsuka,K., and Hartl, F.(Regulation of the Heat-shock Protein 70 Reaction Cycle by the Mammalian DnaJ Homolog, Hsp40. *J.Biol Chem*. **271,** 19617-19624

Multhoff, G., Botzler, C., and Issels, R. (1998) The role of heat shock proteins in the stimulation of an immune response. *Biol Chem*. **379,** 295-300

O'Brien, M. C., and McKay, D. B. (1995) How Potassium Affects the Activity of the Molecular Chaperone Hsc70 .*J.Biol Chem.* **270,** 2247-2250

Old, R. W., Primrose, S. B. (1987) Principles of gene Manipulation. In *An Introduction to genetic Engineering volume , 3rd Edition* .(Carr, N.G, Ingraham, J. L, and Rittenberg, S. C) pp 155,183, 231, Blackwell Scientific Publications

Palleros,D.S., Welch,W.J., and Fink.A.L. (1991) Interaction of hsp70 with unfolded proteins:Effects of temperature and nucleotides on the kinetics of binding. *Proc.Natl.Acad.Sci*.USA. **88**, 5719-5723

Raynes, D.A and Guerriero, V .(1998) Inhibition of Hsp70 ATPase Activity and Protein Renaturation by a Novel Hsp70-binding protein. *J.Biol Chem.* **273,** 32883-32888

Rosenthal, P. J. (1998) Proteases of malaria parasites: New targets for Chemotherapy. *Emerging Infec.disease*. **4**, 1-13

Sharma, Y. (1992) structure and possible function of heat-shock protein in falciparum malaria. *Comp. Biochem. Physiol*. **102,** 4337-44

Sheibani, N. (1999) Prokaryotic Gene fusion expression system and their use in structural and functional studies of proteins, Prep. *Biochem & Biotechnol*. **29(1),** 77-90

Silberg, J. J.,and Vickery,L.E (2000) Kinetic charaterization of the ATPase Cycle of the Molecular Chaperone Hsc66 from *Esherichia coli*. *J.Biol Chem.* **275,** 7779-7786

Smith, D.B., and Johnson, K.S.(1988) Single–step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathinone-S-transferase.*Gene*. **67,** 31-40

Smith, D., Whitesell, L., and Katsanis.E. (1998) Molecular chaperones in biology and Prospects for Pharmacological intervention. *American Soci. Pharmacology*. **50,** 493-500

Southern, M. R., and Lewis, A.P. (1998) JavaShade: multiple sequence alignment box-and-shading on the world wide web. *Bioinformatics*.**14,** 821-822

Suh, W., Burkholder,W.F., Lu, C.Z., Zhao, X., Gottesman. M.E., and Gross,C.A. (1998) Interaction of the Hsp70 molecular chaperone, DnaK with its cochaperone DnaJ. *Prc. Natl. Acad. Sci*.USA. **95**, 15223-15228

The QIA expressionist (1999). A Handbook for high-level expression and purification of 6x His-tagged proteins .pp53 QIAGEN GmbH, Hilden

Tsuji, M., Mattei, D., Nussenzweig, R., Eichinger, D., and Zavala, F. (1994) Demonstration of heat shock protein 70 in the sporozite stage of malaria parasite.*Parasitol. Res*. **80,** 16-21

Uparanukraw, P., Toyoshima, T., Aikawa, M., and Kumar, N. (1993) Molecular cloning and localization of an abundant novel protein of *P.berghei*. *Mol.biochem. Parasitology*. **59,** 223-234

Valero, E., Varon, R., and Garcia-Carmona, F. (2000) Kinetics of a self-

amplifying substrate cycle:ADP-ATP cycle assay. *Biochem.J.* **350,** 237-243

Watanabe, J. (1997) Cloning and characterization of heat shock protein DnaJ homologues from Plasmodium falciparum and comparison with ring infected erythrocyte surface antigen. *Mol.&Biochem Parasitolgy*. **88,** 253-258

Wawrzynow, A., Banecki, B., Wall, D., Liberek, K., Georgopoulos,C., and Zylicz,M. (1995) ATP Hydrolysis Is Required for the DnaJ-dependent Activation of DnaK Chaperone for Binding to Both Native and Denatured Protein Substrates , *J.Biol Chem* . **270 (33),** 19307-19311

Whitley, D., Goldberg, S., and Jordan, N. (1998) Heat shock proteins: A review of the molecular chaperone. *J. Vasc Surg*. **29,** 748-751