

Malarial Molecular Chaperones of the
Plasmodium falciparum Infected
Erythrocyte



Jemma Day
New College
University of Oxford

A thesis submitted for the degree of
Doctor of Philosophy
Trinity 2018

Acknowledgements

First and foremost, I would like to thank Prof. John Vakonakis, the fount of all knowledge, for his supervision over the last 3 and a half years; the advice, guidance and discussions have been invaluable and crucial for the completion of the work presented here. I would also like to thank Prof. Hans-Peter Beck for giving me the opportunity to carry out the cell biology experiments in this thesis at the Swiss Tropical and Public Health Institute and for his advice in general.

My thanks also extend to the Wellcome Trust for funding my research and my DPhil – I am grateful for the opportunity they provided. Additionally, I would like to thank New College for contributions towards travel grants, and for giving me both a home and a community.

To past and present lab mates, thank you for the tea times and office talks as well as the arts and crafts and all the troubleshooting. Thanks to Erin, my malaria mum, for her company in Heidelberg and Lorne, teaching me to clean up after myself in the lab and her kind words whilst writing this thesis. Jodie and Julia made coming to the lab even on the difficult days so much easier with their support and co-procrastination. Thank you also to Natassa and Beatrice for your post-doc wisdom on all things lab- and writing-related as well as the language lessons – ευχαριστω! 谢谢! Dangge to everyone at the Swiss TPH for welcoming me into the lab and making me feel at home for the few months I spent there. In particular, thank you to Bea, Armin and Jan for their help and patience.

The friends I have met in Oxford, both in the lab and outside, have made the last four years a truly enjoyable experience. They have helped me more than they know with support in various ways: from cat sushi Mondays to Friday night Maxwell's; the spoon dancing at Juxon Street and the pints at the Half Moon; from guest nights galore to the late-night cheesy chips. My housemates in particular are the weirdest and most wonderful people I could have ever hoped to meet; Josh, MPD, lovely Leah, Caroline and, perhaps most importantly, Triangle.

I cannot thank my family enough for their support and the pride they have in me without ever putting me under pressure. My parents worked hard to provide myself and my brother with opportunities that they did not have and, through this, taught me the value of hard work. Thank you to my brother for being the best of all the big brothers.

Above all, thanks Ilya.

Abstract

Resistance to common antimalarials is growing, resulting in the need to develop new therapeutics with the aim of disease elimination. Here we present the malarial molecular chaperones of the *Plasmodium falciparum* infected erythrocyte as potential drug targets and provide structural information for structure-based drug design. The parasite exports a single Hsp70 into the erythrocyte, PfHsp70-x, which has been linked to virulence. PfHsp70-x colocalises in J-dots with two parasite Hsp40 proteins – PFA0660w and PFE0055c. PFA0660w is a specific stimulator of PfHsp70-x whilst PFE0055c can also stimulate the activity human chaperones.

In this thesis, it was shown that PfHsp70-x is important in the parasite's response to heat shock at certain stages of the intraerythrocytic life cycle. The ATPase domain of PfHsp70-x was structurally determined using X-ray crystallography and a unique druggable pocket was discovered. Numerous commercially available Hsp70 inhibitors were found to successfully inhibit the basal and/or the Hsp40 stimulated activity of PfHsp70-x. A combination of X-ray crystallography, biochemical assays and biophysical methods were employed to characterise the interaction of PfHsp70-x with PFA0660w and PFE0055c to understand the specificity of stimulation.

The role of PfHsp70-x in protection and proliferation of the parasite, alongside the inhibition of PfHsp70-x by available Hsp70s inhibitors, demonstrates the possibility of repurposing chaperone inhibitors as antimalarials. Differential interactions of PfHsp70-x with its cochaperones provide a route for specifically targeting PfHsp70-x as Hsp70 proteins require stimulation by a Hsp40. The work presented here characterises just a part of a wider network of chaperones likely formed of parasite proteins and hijacked human chaperones.

Contents

List of Figures	vii
List of Tables	x
List of Abbreviations	xi
1 Introduction	1
1.1 Background	1
1.2 <i>Plasmodium falciparum</i>	2
1.2.1 Intraerythrocytic life cycle and pathogenicity	5
1.3 Molecular Chaperones	7
1.3.1 The Hsp70 family	8
1.3.2 Cochaperones of Hsp70 - the Hsp40 family	13
1.4 Molecular chaperones of <i>P. falciparum</i>	18
1.4.1 PfHsp70-x – the exported Hsp70	19
1.4.2 J dot resident PfHsp40s	25
1.5 Molecular chaperones as drug targets	25
1.5.1 Malarial chaperones - an emerging therapeutic target	26
1.6 Aims and work presented	27
2 Methods	28
2.1 Cloning	29
2.2 Protein Expression and Purification	30
2.2.1 PfHsp70-x, Hsc70, PFA0660wJ, PFE0055cJ	30
2.2.2 DnaJ	33
2.3 NMR	34
2.3.1 HSQC Titrations	34
2.3.2 Backbone Assignments	35
2.3.3 Saturation Transfer Difference	35
2.4 Biophysics and Biochemical Assays	36
2.4.1 Mass Spectrometry	36
2.4.2 Circular Dichroism	36
2.4.3 MESG ATP Hydrolysis Assay	37

2.5	X-Ray Crystallography	37
2.5.1	Structure Determination of PfHsp70-x _{ATPase} :AMP-PNP . . .	37
2.5.2	Structure Determination of PfHsp70-x _{ATPase} :ADP	38
2.5.3	Inhibitor Soaking of PfHp70-x _{ATPase} Crystals	38
2.5.4	Structure Determination of PFA0660w _J	39
2.6	Computational methods	39
2.6.1	Homology Modelling	39
2.7	<i>In vitro</i> Plasmodium falciparum Assays	40
2.7.1	Parasite Culturing	40
2.7.2	PfHsp70-x knockdown: induction and validation	41
2.7.3	Stress Assays	41
2.7.4	Drug Assays	43
2.8	Graphs and Structures	43
3	Cell Biology	44
3.1	Contributions to Data	44
3.2	Introduction	45
3.3	PfHsp70-x knockout and knockdown cell lines	46
3.4	Δ PfHsp70-x susceptibility to antimalarials	48
3.5	Bromo- β Lapachol - the 'specific inhibitor of PfHsp70-x	53
3.6	The effect of stress on PfHsp70-x knockdown parasite growth	55
3.6.1	Oxidative stress	55
3.6.2	Heat stress	56
3.6.3	Discrepancies between biological repeats	58
3.6.4	Heat stress through the parasite's life cycle	65
3.7	Discussion	68
4	PfHsp70-x	73
4.1	Crystal Structure of PfHsp70-x _{ATPase}	73
4.1.1	Comparison with human Hsp72	82
4.1.2	The crystal structure of PfHsp70-x _{ATPase} :ADP	85
4.2	HSQC NMR of the PfHsp70-xATPase nucleotide cycle	90
4.3	Discussion	95
5	Inhibiting PfHsp70-x	98
5.1	The ATPase activity of PfHsp70-x	98
5.2	Testing Hsp70 inhibitors for PfHsp70-x inhibition	102
5.3	The mechanism of inhibition	110
5.4	Studying ligand binding with STD-NMR	113
5.5	Discussion	118

6	PfHsp70-x and cochaperones PFA0660w and PFE0055c	125
6.1	The J domain of PFA0660w encodes specificity	126
6.2	Crystal structure of PFA0660w _J	127
6.3	Homology model of PFE0055c _J	135
6.4	The J domain - PfHsp70-x interaction	141
6.5	Discussion	152
7	Discussion	156
7.1	Molecular chaperones as antimalarial targets	157
7.2	Druggability of PfHsp70-x _{ATPase}	159
7.3	Targeting the PfHsp70-x/Hsp40 complex	164
7.4	Other exported PfHsp40s	165
7.5	The infected erythrocyte chaperone network	167
7.6	Future work	168
7.7	Conclusions	170
Appendices		
A	Construct cloning primers	172
B	Generation of PFA0660w knockdown parasite lines	174
B.1	Construct cloning	174
B.2	Transfection	176
B.3	Current status	178
References		179

List of Figures

1.1	Progress towards elimination of malaria has stalled	2
1.2	The life cycle of <i>P. falciparum</i>	4
1.3	Domain architecture and structure of a canonical Hsp70	10
1.4	The nucleotide cycle of the Hsp70 machinery	11
1.5	Classification of Hsp40 cochaperones	15
1.6	The DnaK-DnaJ complex	17
1.7	<i>P. falciparum</i> Hsp70s	19
1.8	Colocalisation of PfHsp70-x with J dot Hsp40s and PfEMP1	21
2.1	SDS PAGE of purified PfHsp70-x FL and ATPase constructs	32
2.2	SDS PAGE of purified PFA0660w and PFE0055c J domains	33
3.1	The GlmS conditional knockdown system	47
3.2	Δ PfHsp70-x confirmation	48
3.3	Antimalarial drug experiment schematic	50
3.4	Dose-response curves for 8 h antimalarial treatment	52
3.5	Dose-response curves for 1 h antimalarial treatment	53
3.6	Dose-response curves for BBL treatment	54
3.7	Oxidative stress growth experiment	56
3.8	Heat stress growth experiment schematic	57
3.9	Oxidative stress growth experiment variability	59
3.10	Heat stress growth experiment variability	60
3.11	Flow cytometry of <i>P. falciparum</i> parasites	62
3.12	DNA content of <i>P. falciparum</i> in the intraerythrocytic life cycle	63
3.13	The effect of heat stress on life cycle progression	64
3.14	Initial population make-up of asynchronous cultures	65
3.15	Heat stress growth experiment with synchronised parasites	67
3.16	PfHsp70-x expression levels during intraerythrocytic development	70
4.1	Asymmetric unit interactions of PfHsp70-x _{ATPase} :AMP-PNP	75
4.2	PfHsp70-x _{ATPase} :AMP-PNP crystal structure	77
4.3	AMP-PNP ligand	79
4.4	AMP-PNP ligand 3D	80

4.5	B-factors of PfHsp70-x _{ATPase} :AMP-PNP	81
4.6	Canonical Hsp70 _{ATPase} architecture	83
4.7	Comparison of PfHsp70-x and human Hsp72	84
4.8	Non-conserved patches of PfHsp70-x _{ATPase}	85
4.9	Comparing PfHsp70-x _{ATPase} :AMP-PNP and PfHsp70-x _{ATPase} :ADP	88
4.10	Comparison of PfHsp70-x _{ATPase} ADP and AMP-PNP binding	89
4.11	¹ H- ¹⁵ N HSQC spectra of PfHsp70-x _{ATPase} with nucleotides	92
4.12	¹ H- ¹⁵ N HSQC spectra of PfHsp70-x _{ATPase} with ADP and AMP-PNP	93
4.13	The effect of Mg ²⁺ on ATPase domain structure.	94
5.1	MESG assay	100
5.2	PfHsp70-x ATP hydrolysis	101
5.3	Inhibition of PfHsp70-x ATP hydrolysis activity	105
5.4	Stimulated PfHsp70-x inhibition by methylene blue and BBL	107
5.5	Stimulated PfHsp70-x inhibition by dihydropyrimidines	108
5.6	Stimulated PfHsp70-x inhibition by MKT-077 and YM-01	109
5.7	Cysteines of PfHsp70-x _{ATPase}	112
5.8	Lack of cysteine modification upon methylene blue treatment	113
5.9	Schematic of saturation transfer difference NMR	114
5.10	1D ¹ H spectrum of methylene blue	115
5.11	1D ¹ H spectrum of BBL	116
5.12	STD-NMR with methylene blue	117
5.13	STD-NMR with BBL	118
5.14	Summary of stimulated and unstimulated PfHsp70-x inhibition	119
5.15	115-7c predicted binding site	122
5.16	MKT-077 predicted binding site	123
6.1	J domain stimulation	128
6.2	Asymmetric unit of PFA0660w _J crystal structure	131
6.3	The crystal structure of PFA0660w _J	133
6.4	A comparison of J domain structures	134
6.5	Mapping J domain similarities	135
6.6	¹ H- ¹⁵ N HSQC PFE0055c _J assignment	137
6.7	Secondary structure analysis of PFE0055c _J using PECAN	138
6.8	¹ H- ¹⁵ N HSQC PFA0660w _J assignment	139
6.9	Secondary structure analysis of PFA0660w _J using PECAN	140
6.10	NMR of PFA0660w _J with PfHsp70-x and Hsc70	143
6.11	NMR of PFE0055c _J with PfHsp70-x and Hsc70	144
6.12	NMR titrations of cochaperones with chaperones	145
6.13	Peak intensity reduction by residue	147

6.14	Mapping perturbations onto J domain structures	148
6.15	The hydrophobic patch of PFE0055c _J	149
6.16	Hybrid model of PfHsp70-x and cochaperones	151
6.17	Summary of cochaperone residues affected by PfHsp70-x binding . .	153
7.1	Druggable pockets	161
7.2	Pocket II proximity to J domain binding site	162
7.3	Conservation of druggable pockets	164
7.4	Exported Hsp40s of <i>P. falciparum</i>	167
7.5	The chaperone network of the <i>P. falciparum</i> infected erythrocyte .	168
B.1	The pCRISPR-HADD2A-PFA0660w plasmid	176

List of Tables

2.1	Table of constructs	30
4.1	PfHsp70-x _{ATPase} :AMP-PNP structure statistics	76
4.2	PfHsp70-x _{ATPase} :ADP structure statistics	87
5.1	Hsp70 inhibitors	103
5.2	Solvent accessibility of PfHsp70-x _{ATPase} cysteines	111
6.1	PFA0660w _J structure statistics	130
A.1	Table of primers	173
B.1	Table of primers for pCRISPR-HADD2A-PFA0660w	177

List of Abbreviations

ADP		Adenosine Diphosphate
AMP-PNP	. .	5'-adenylyl-imidodiphosphate
ATP		Adenosine Triphosphate
BBL		Bromo-B-Lapachol
Cas		CRISPR-associated
CD		Circular Dichroism
CRISPR	. . .	Clustered Regularly Interspaced Short Palindromic Repeats
Hsp		Heat shock protein
HSQC		Heteronuclear Single Quantum Coherence
MB		Methylene Blue
MESG		2-amino-6-mercapto-7-methylpurine ribonucleoside
NMR		Nuclear Magnetic Resonance
PDB		Protein Data Bank
PECAN		Protein Energetic Conformational Analysis
PfEMP1		<i>Plasmodium falciparum</i> Erythrocyte Membrane Protein 1
PHIST		<i>Plasmodium</i> Helical Interdispersed Sub-telomeric
PISA		Protein Interface, Surfaces and Assemblies
PNPase		Purine nucleoside phosphorylase
PV		Parasitophorous vacuole
RBC		Red Blood Cell
RMSD		Root-Mean-Square Difference
SBD		Substrate Binding Domain
STD		Saturation Transfer Difference

1

Introduction

Contents

1.1	Background	1
1.2	<i>Plasmodium falciparum</i>	2
1.2.1	Intraerythrocytic life cycle and pathogenicity	5
1.3	Molecular Chaperones	7
1.3.1	The Hsp70 family	8
1.3.2	Cochaperones of Hsp70 - the Hsp40 family	13
1.4	Molecular chaperones of <i>P. falciparum</i>	18
1.4.1	PfHsp70-x – the exported Hsp70	19
1.4.2	J dot resident PfHsp40s	25
1.5	Molecular chaperones as drug targets	25
1.5.1	Malarial chaperones - an emerging therapeutic target	26
1.6	Aims and work presented	27

1.1 Background

In 2016, malaria caused 445,000 deaths from 216 million cases worldwide [1]. Nearly half of the world's population is at risk of malaria with the vast majority of cases and deaths occurring in sub-Saharan Africa. Children under the age of 5 and pregnant women are particularly at risk of developing severe malaria [2]. The incredible progress previously made towards malaria elimination has recently stalled with 5 million more cases in 2016 than 2015 and a similar number of

deaths (446,000 in 2015) (Fig. 1.1). Numerous socio-economical factors, lack of effective vaccines and emerging drug resistance all contribute to preventing further progress towards eradication [3].

A wide variety of symptoms result from malaria with the disease categorized as uncomplicated or severe [4]. A patient with uncomplicated malaria experiences fever, headaches and nausea whereas severe cases can lead to cerebral malaria causing seizures or coma, acidosis, severe anaemia and organ failure. Malaria is caused by protozoan *Plasmodium* parasites which are transmitted through the bites of female *Anopheles* mosquitoes. Five parasite species cause malaria in humans with *Plasmodium falciparum* being by far the most lethal [5].

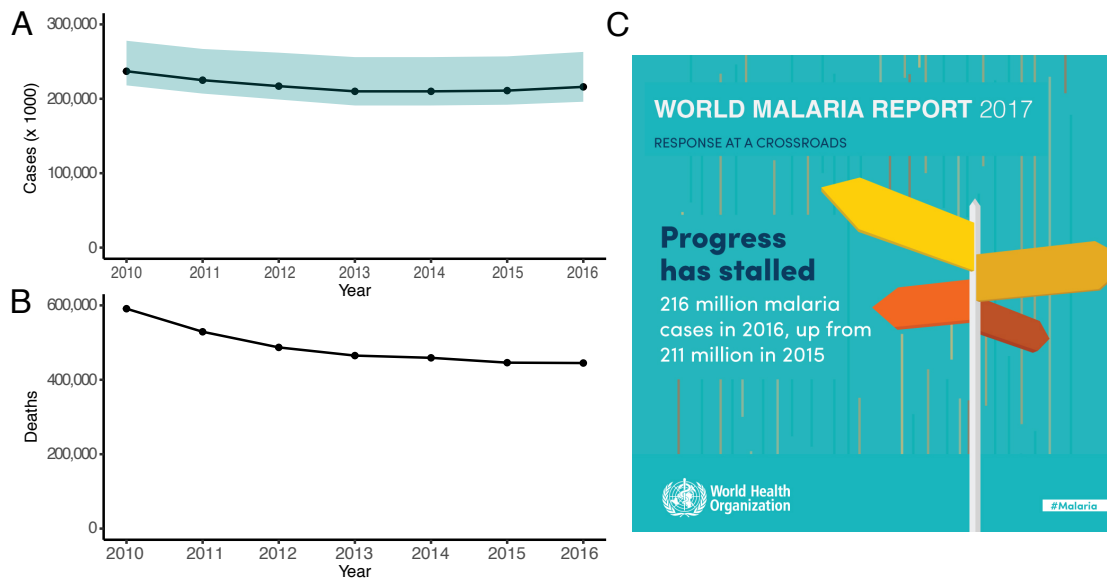


Figure 1.1: Progress towards elimination of malaria has stalled. **A:** Malaria cases per year 2010 - 2016. The upper and lower estimates of cases are indicated by the cyan ribbon. **B:** Number of malaria related deaths per year 2010 - 2016. **C:** The World Malaria Report 2017 from WHO highlights concern over the stalling of progress [1].

1.2 *Plasmodium falciparum*

P. falciparum has a complex life cycle in which it inhabits two separate hosts; the *Anopheles* mosquito and the human. The life cycle can be simplified into three stages; the mosquito stage, the human liver stage and the human blood stage (Fig. 1.2A).

The mosquito stage of the life cycle begins as the female *Anopheles* takes a blood meal from an infected person ingesting parasite gametocytes. In the mosquito gut, sexual reproduction of the parasite occurs to form an ookinete. The ookinete is able to penetrate the midgut wall and develop into an oocyst. Thousands of sporozoites develop inside the oocyst during the process of sporogony and are released upon oocyst lysis. The highly motile sporozoites then travel to the salivary gland allowing transmittance of the parasite during the next blood meal through the mosquito's saliva.

Injected sporozoites travel through the blood stream to the liver where they infect hepatocytes thus beginning the liver stage of infection. Sporozoites replicate and mature into schizonts, which rupture and release merozoites back into the blood stream. After the liver stage, merozoites infect erythrocytes and so begins the asexual parasite multiplication of the blood stage.

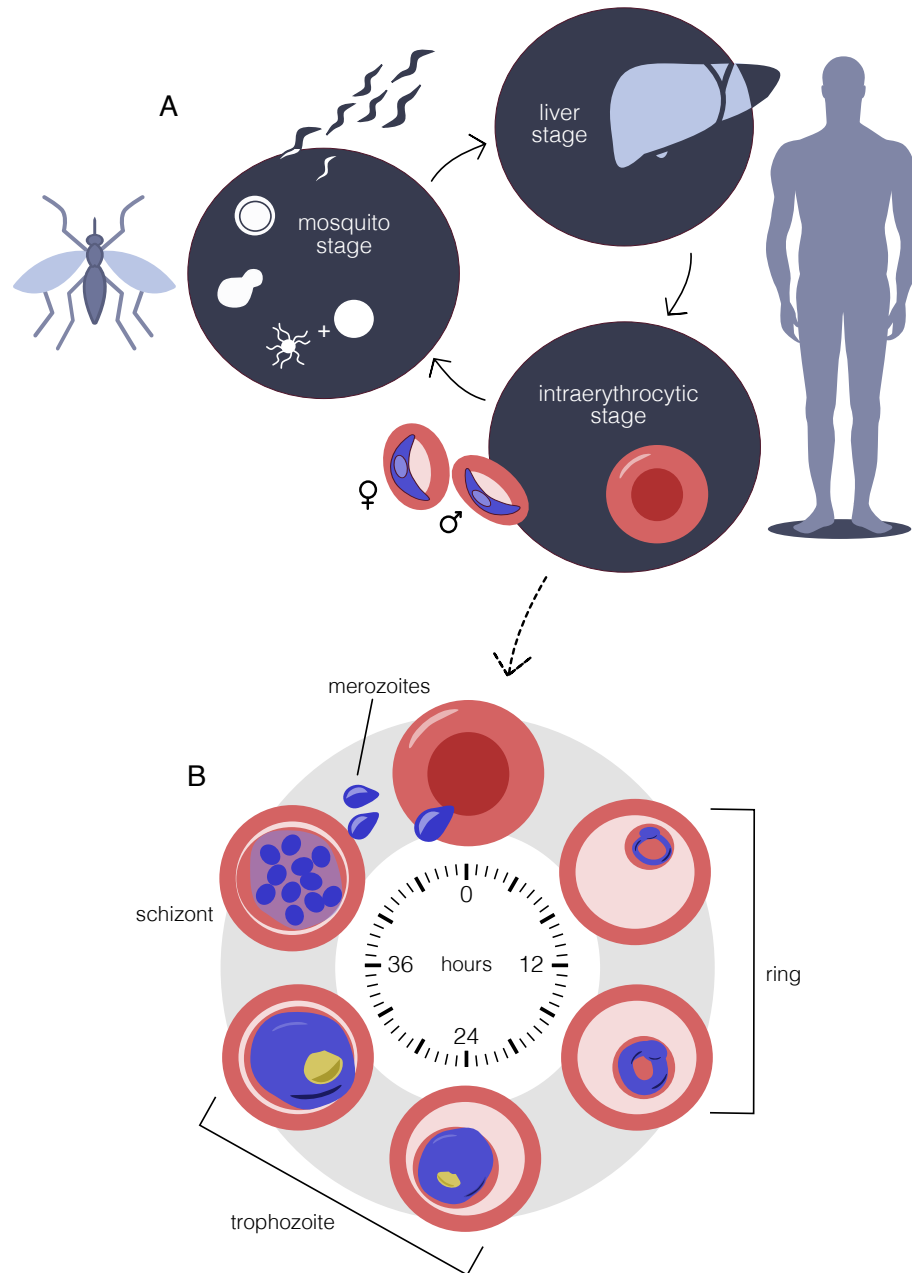


Figure 1.2: The life cycle of *P. falciparum*. **A:** The three stage life cycle of *P. falciparum*. **B:** A detailed view of the intraerythrocytic asexual stage of the parasite. The development of a single parasite within a red blood cell is shown over 48 hours - a full cycle. The parasite progresses from an merozoite invading the red blood cell into the ring stage then trophozoite stage followed by the schizont stage. The cycle ends with the rupture of the schizont causing release of up to 32 merozoites. The released merozoites can then invade new erythrocytes and thus continue to proliferate asexually. The parasitophorous vacuole (pink) can be seen to expand as the parasite grows until both fill the erythrocytes in the late stages of the life cycle.

1.2.1 Intraerythrocytic life cycle and pathogenicity

The blood stage of *P. falciparum* is responsible for the clinical manifestations of the disease and so targeting this stage can be a useful strategy in treating malaria.

Initially, a merozoite invades an erythrocyte and quickly develops into the ring stage, named as such due to the appearance of this stage as seen by light microscopy with Giemsa staining (Fig. 1.2B). In the ring stage, the parasite expresses and exports a large number of proteins into the erythrocyte cytoplasm (the exportome). After 24 hours, the ring stage parasite evolves into a trophozoite. The trophozoite stage is characterized by rapid increase in parasite size and DNA content in addition to the presence of hemozoin (a crystallized form of heme which is released by hemoglobin digestion). Finally, the parasite enters the schizont stage at approximately 36 hours post invasion. During schizogony, up to 32 merozoites are generated and fill the infected erythrocyte [6]. The host cell is ruptured 48 hours post invasion releasing the next generation of merozoites which will invade new erythrocytes and continue the blood stage life cycle. The rupturing of infected erythrocytes leads to anaemia and the release of parasitic proteins upon rupture triggers fever. A small portion of blood stage parasites will commit to sexual development and form gametocytes enabling transmission to mosquitos and thus to other humans [7].

Erythrocyte Remodelling

During the intraerythrocytic lifecycle of *P. falciparum* infection, the infected erythrocyte undergoes major changes induced by the parasite. Upon invasion of the merozoites into the erythrocyte, the parasite forms a tightly fitting vacuole termed the parasitophorous vacuole (PV) [8]. The PV is thought to be created from the erythrocyte membrane but is further altered and expanded by lipids secreted from parasite organelles, termed rhoptries, as the parasite itself grows [9, 10, 11]. The PV forms the parasite-host cell interface during the intraerythrocytic stage of infection and is crucial for controlling the passage of nutrients, disposing of waste products and in exporting parasitic proteins in to the host cell. Our current

understanding of parasite protein export involves the secretion of proteins across the parasite membrane into the PV through the canonical secretory pathway of the parasite (endoplasmic reticulum, Golgi apparatus, vesicles) followed by fusion of vesicles with the parasite plasma membrane [12, 13]. Proteins destined for the host cell then must cross the PV membrane. The export of parasitic proteins across the PV membrane has been shown to be mediated by the *Plasmodium* translocon of exported proteins (PTEX) [14]. PTEX is a membrane protein complex that unfolds and transports proteins in an ATP-dependent process [15, 16]. It is believed to be located in the PV membrane to allow the export of proteins from the PV into the host cell [17]. Once in the host cell cytoplasm, the exported protein is further targeted to particular sites through signal sequences or interactions with binding partners.

The parasitic exported proteins, also known as the exportome, extensively remodel the erythrocyte by inducing changes in cell rigidity and the formation of cytoadherent knobs on the cell surface along with additional changes. *P. falciparum* infected erythrocytes cytoadhere to endothelial cells in the microvasculature and to uninfected erythrocytes forming rosettes. Immobilisation of infected erythrocytes prevents their clearance by the spleen thereby prolonging infection. Cytoadhesion of infected erythrocytes can lead to the blocking of microvasculature which, when occurring in the brain, can cause coma and eventually be fatal, potentially explaining the lethality of *P. falciparum* infections [18].

During the blood stage of infection, the parasite and its host cell are exposed to numerous stresses: episodes of fever characteristically recurring every 48 hours raise the host temperature up to 41 °C; digestion of hemoglobin for use as a source of nutrition releases soluble heme which can create free radicals [19]; the host's immune response generates reactive oxygen species to attack pathogens [20]. The parasite survives such stresses and in fact proliferates, which indicates some mechanism of protection. The malaria parasite, as with all organisms, possesses an arsenal of proteins whose role it is to protect the cell against such stresses. Such proteins are named molecular chaperones.

1.3 Molecular Chaperones

Molecular chaperones are responsible for maintaining protein homeostasis and assisting *de novo* folding within the cell whether in normal physiological conditions or under stress [21]. The folding of proteins into their native state is crucial for their function and, thus, protein quality control is critical in cellular survival and proliferation. In times of stress, such as by high temperatures, a protein's fold can be compromised and may lead to misfolding or aggregation. Molecular chaperones are able to associate with such compromised proteins to prevent misfolding or aggregation, actively refold proteins or even target unsalvageable proteins for degradation. In addition to their role in stress, molecular chaperones also play important roles in routine *de novo* folding of large, complex multidomain proteins, in assembly of macromolecular complexes, the translocation and trafficking of proteins and complexes, and in maintenance of intrinsically disordered proteins [22]. Collapse of protein homeostasis can lead to the accumulation of misfolded and oxidised proteins in addition to the deposition of aggregates resulting in cell degeneration. Numerous diseases have been shown to be attributed to protein misfolding including cystic fibrosis, alpha1-antitrypsin deficiency, type 2 diabetes and neurodegenerative conditions such as Parkinson's disease, Alzheimer's disease and Huntington's disease [23]. Thus, molecular chaperones and their maintenance of cellular proteins are critical for an organism's health.

The molecular chaperone family was first discovered by the induction of certain protein's expression in *Drosophila* [24, 25, 26]. These proteins were thus named heat shock proteins (Hsp) and were found to have roles in protein folding leading to the discovery of stress-induced molecular chaperones. Hsp's are subdivided into families initially based on their molecular weight: Hsp100, Hsp90, Hsp70, Hsp60, Hsp40 and small Hsps. More recently, chaperones are grouped by common functions and mechanisms rather than just molecular weight [27]. Molecular chaperones from different groups work together in a complex network along with cochaperones such as nucleotide exchange factors to maintain proteostasis. In this thesis we focus

primarily on proteins from the Hsp70 and Hsp40 groups, so details of the other molecular chaperone classes will not be discussed here.

1.3.1 The Hsp70 family

The Hsp70 family makes up a major portion of the chaperone network. Members of the family are highly conserved and ubiquitously found in all organisms from bacteria to humans [28]. The functions of Hsp70 proteins are wide and varied [29] but include protein translocation, nascent protein folding, macromolecular complex association and aggregation prevention. Central to these functions are Hsp70 protein's ability to act as both a 'holdase' and a 'foldase'. Hsp70 proteins can bind and hold (hence holdase) to aggregation-prone proteins by interactions with exposed hydrophobic residues thereby preventing the exposed hydrophobic regions from associating and forming dangerous aggregates. Additionally, Hsp70 proteins are able to bind non-native proteins and induce their folding into the native state although this exact mechanism remains unclear. The expression of Hsp70 proteins is induced in response to stress but a subsection of the Hsp70 family, the heat shock cognate (Hsc) 70 group, are constitutively expressed and necessary for general housekeeping and day-to-day cellular functions. Often, Hsp70 proteins are found in discrete locations within the cell where they have evolved specialist functions to cater for client proteins in that subcellular compartment [30]. The functions of Hsp70 proteins are further expanded and specialised by their interactions with the rest of the chaperone network. The crucial role of Hsp70s in the regulation of protein homeostasis and response to stress, alongside its implication in diseases such as cancer and neurodegeneration, have led to Hsp70s emerging as attractive potential drug targets [31].

Canonical Hsp70 proteins consist of an N-terminal ATPase domain, also known as the nucleotide binding domain, and a C-terminal substrate binding domain joined by a highly conserved hydrophobic linker [32] (Fig. 1.3). The ATPase domain is composed from four subdomains that form a deep crevice into which ATP binds and is hydrolysed [33]. The substrate binding domain is divided into two subdomains; a

twisted beta-sandwich (SBD β) to which the hydrophobic residues of client proteins bind and an alpha-helical lid (SBD α). The SBD recognises a short degenerate motif consisting of 5 hydrophobic residues flanked by positively charged amino acids [34]. Such motifs are present every 30-40 residues in virtually all proteins, but they are usually hidden within the hydrophobic core unless the protein is denatured or just exiting the ribosome and yet to fold.

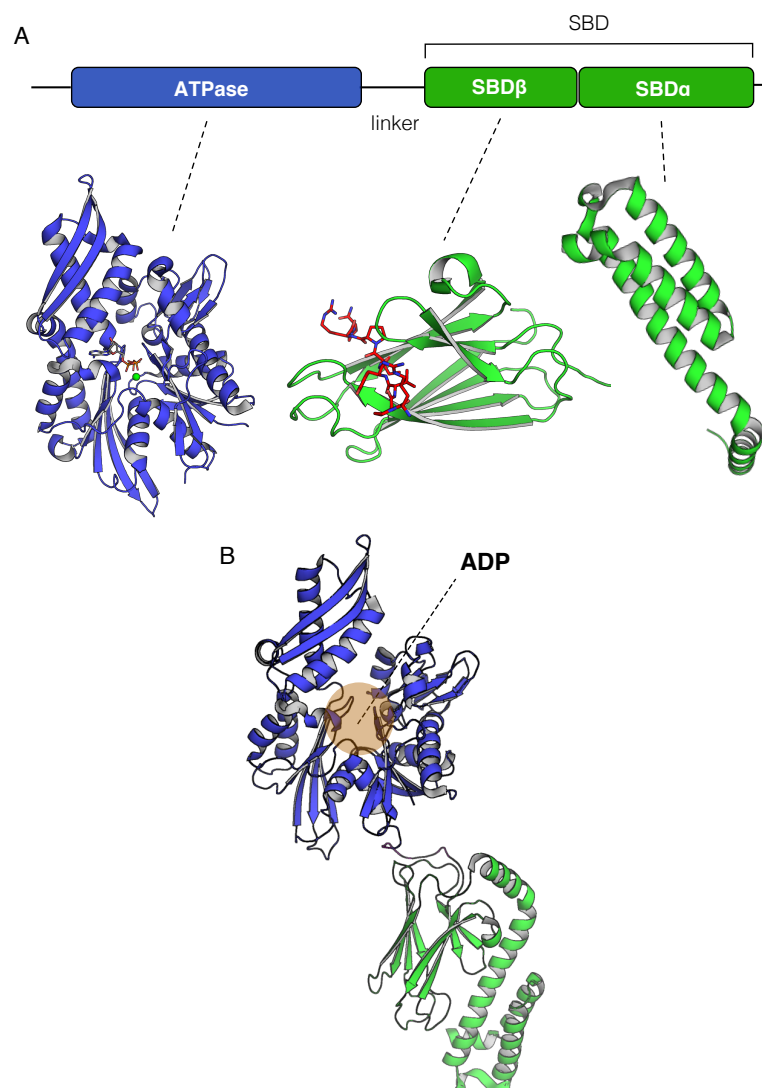


Figure 1.3: Domain architecture and structure of a canonical Hsp70. **A:** Domain organisation of Hsp70 proteins and associated domain folds. At the N-terminus is the nucleotide binding domain, also known as the ATPase domain for its ATP hydrolysis activity. The C-terminus contains the substrate binding domain (SBD) which in turn is made up from a twisted beta-sheet (SBDβ) and an alpha-helical lid (SBDα). The two domains are joined by a conserved linker. The ATPase domain from *E.coli* DnaK is shown to demonstrate the typical fold of an Hsp70 ATPase domain (PDB 5NRO) [35] with ATP (orange, stick representation) and an Mg²⁺ ion (green, sphere representation). Both the SBDα and the SBDβ from DnaK are shown with the SBDβ in complex with a model peptide (red) (PDB 4E81) [36]. **B:** The solution structure of full-length DnaK bound to ADP created by combining X-ray crystallography structures with NMR residual dipolar coupling data (PDB 2KHO) [37]. The ATPase domain is coloured blue and the SBD is coloured green. For molecules shown in stick representation, such as ATP and the model peptide, atoms are coloured according to element: nitrogen in blue; oxygen in red; sulphur in yellow. This colouring is used throughout.

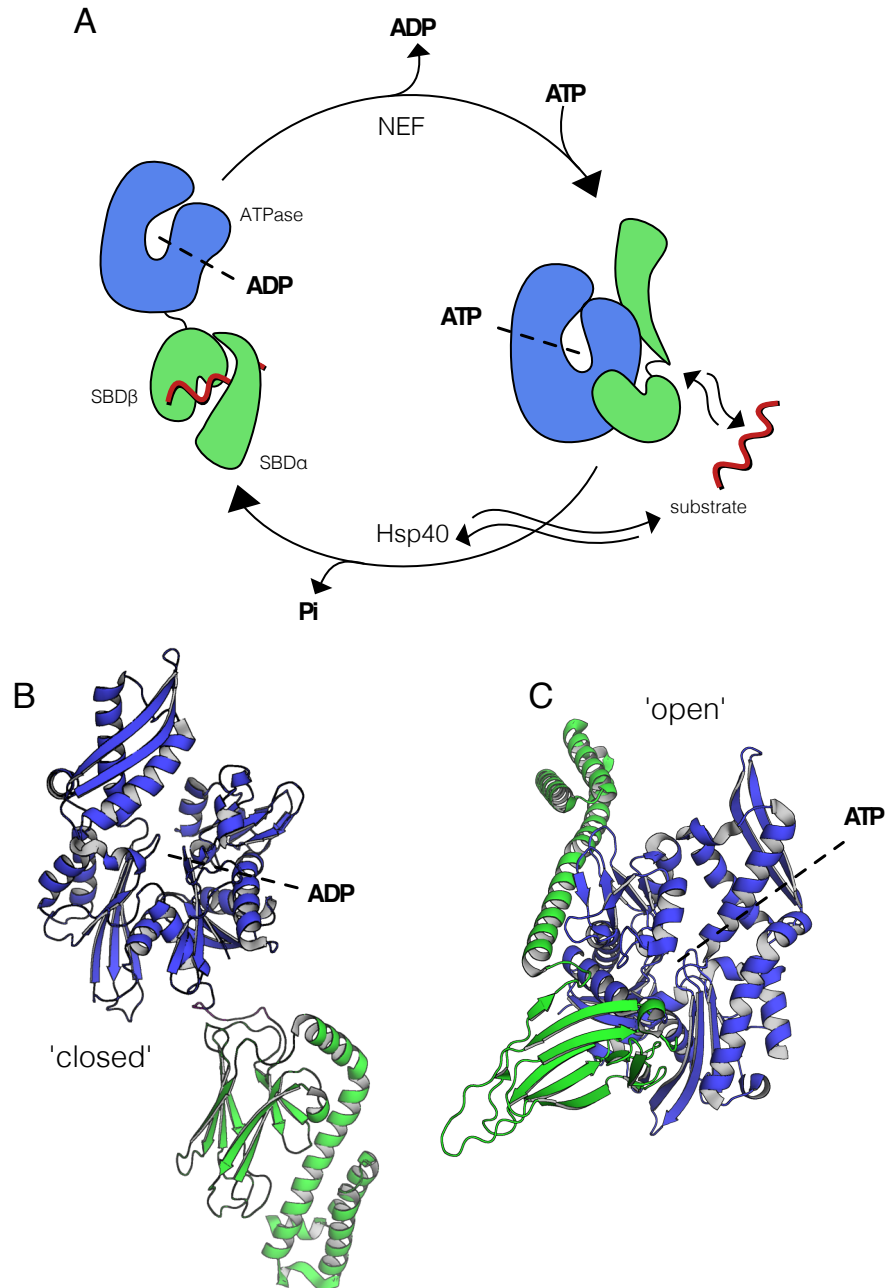


Figure 1.4: The nucleotide cycle of the Hsp70 machinery. **A:** A diagram illustrating the nucleotide cycle of Hsp70. Hsp40 proteins promote ATP hydrolysis causing large-scale conformational changes which cause the substrate to become trapped. Upon ADP removal by a nucleotide exchange factor (NEF), the Hsp70 can rebind ATP and resume the 'open' conformer allowing the release of the folded protein and the binding of new substrates. **B:** The crystal structure of full-length DnaK in the ADP 'closed' state in relation to the SBD (PDB 2KHO) [37]. **C:** The crystal structure of full-length DnaK in the ATP 'open' state in relation to the SBD (PDB 4JNE) [38].

The molecular mechanism of Hsp70 proteins binding to and releasing client proteins involves large scale conformational changes linked to the nucleotide cycle of the ATPase domain and is driven by allosteric communication between the two domains via the interdomain linker (Fig. 1.4) [32, 39]. Recently solved crystal structures of full-length Hsp70 proteins alongside NMR, limited proteolysis, tryptophan fluorescence and small-angle X-ray scattering studies have allowed insights into the chaperone cycle of Hsp70 [40, 41, 42]. In the ADP-bound state, also known as the closed conformation, the SBD α lays across the SBD β acting as a lid forming a pocket into which the extended substrate is trapped [43, 37]. The closed conformation of Hsp70 has low substrate exchange rates but higher substrate affinity. Upon ATP binding, the Hsp70 adopts an open conformation allowing client protein binding [44]. In the ATP bound state, the lobes of the ATPase domain rotate in relation to each other to remodel the surface allowing the docking of both the interdomain linker and the SBD leading to the opening of the SBD in a scissor-like fashion [38]. It is in this state that the Hsp70 binds the substrate protein due to high association rates yet low affinity. Hydrolysis of ATP occurs through nucleophilic attack on the γ -phosphate by a well-positioned water molecule. The water is positioned by glutamate, aspartate and threonine residues within the active site and a lysine residue coordinates the γ -phosphate itself to stabilise the transition state. The positioning of these catalytic residues in the active site are affected by the rotation of the ATPase domain lobes which means anything that may externally affect lobe rotation will change the rate of ATP hydrolysis. Both client protein binding to the SBD and binding of the cochaperone Hsp40 have been shown to induce such rotations to better align catalytic residues and therefore stimulate ATP hydrolysis [45].

The basal ATP hydrolysis of Hsp70 proteins is low with turnover numbers of 1 ATP molecule per 20-30 minutes, therefore stimulation by synergism with substrates and Hsp40 proteins is a crucial part of the chaperone cycle (Fig. 1.4A) [46, 47]. Nucleotide exchange factors also play an important role in the chaperone cycle as they remove ADP post-ATP hydrolysis, thereby allowing ATP to rebind

causing the substrate to be released and allows the cycle to continue [48, 49]. It is thought that client protein binding induces changes in the SBD that expose and reposition loops carrying ATPase domain interacting residues, thereby increasing the ability of the SBD to dock to the ATPase domain and thus induce lobe rotation; in this manner substrate binding affects ATP hydrolysis [50]. The stimulation of ATPase activity by client binding protein provides a way for the chaperone to increase its activity in a high concentration of denatured proteins, for example in a time of stress, to maintain proteostasis.

An important and unresolved part of the Hsp70 mechanism is exactly how the chaperone acts on its substrate protein to cause refolding of denatured or aggregated proteins, translocation of proteins across membranes and assembly of complexes. There are several models that attempt to explain how Hsp70 proteins are able to bind to both folded proteins (in aggregates, complexes and for translocation) and unfolded proteins (nascent and denatured) and how they are able to induce correct folding or disaggregation, but the field is far from a definitive answer. Current models include conformer selection, induced unfolding and entropic pulling with all models relying on the highly dynamic nature of the Hsp70 chaperone and the intrinsic fluctuations of a substrate protein's structure; it is possible that Hsp70 functions through a combination of the aforementioned models [51].

The activity of Hsp70 proteins is highly regulated and carefully controlled in the cell. The intrinsically low basal ATPase activity of Hsp70 proteins is stimulated by client binding, as previously discussed, and by the cochaperones Hsp40s. Hsp40 proteins not only stimulate the ATPase activity of Hsp70s but also impart substrate specificity by delivering client proteins [49].

1.3.2 Cochaperones of Hsp70 - the Hsp40 family

Like Hsp70s, the Hsp40 family of proteins is found ubiquitously in all organisms. The family is characterised by the presence of the J domain and thus members are often called J proteins as many Hsp40 proteins exceed 40 kDa in molecular weight. Excluding the characteristic J domain, Hsp40 proteins are highly diverse in

sequence and function. Organisms have gained versatility of the Hsp70 chaperone machinery not through amplification and diversification of Hsp70 genes but through Hsp40 proteins instead; for example, humans have only 11 Hsp70 proteins but an arsenal of 41 Hsp40s [52, 53].

The defining feature of Hsp40 proteins, the J domain, is a highly conserved domain of 70 amino acids (Fig. 1.5B). J domains consist of 4 alpha-helices with two main helices (II and III) running antiparallel to each other. Within this domain is the invariable histidine, proline and aspartate (HPD) motif which lays between the two main helices on an extended loop. This motif has been shown to be crucial for stimulating the ATPase activity of Hsp70s through interactions with the ATPase domain and interdomain linker of the Hsp70 [49].

Outside the J domain, Hsp40 proteins are extremely dissimilar and contain a combination of additional domains that impart specific functions on the cochaperone [54, 49]. Hsp40 proteins have been classified into three groups by their domain organization (type I, II and III) with type I based on the architecture of DnaJ – an *E. coli* Hsp40 (Fig. 1.5A) [55, 56, 57]. A type I Hsp40 has an N-terminal J domain, followed by a region rich in glycine and phenylalanine amino acids (G/F), a zinc finger motif consisting of four cysteine-rich repeats (ZF), and a client protein binding domain at the C-terminus (CTD) [58, 59, 60]. At the extreme C-terminus of the Hsp40 is a dimerization domain which leads to an increased affinity for clients and Hsp70s (Fig. 1.5C)[61, 62]. Type II Hsp40s are similar to type I but lack the zinc-finger domain. Type III Hsp40s contain a J domain that can be located anywhere within the protein, not just at the N-terminus. Often an additional group, type IV, is added as members contain a J domain however the HPD, a crucial tripeptide motif, is mutated suggesting this group does not function like canonical Hsp40s. This classification does not relate to function or mechanism however as even within classes Hsp40s are very diverse which allow them to drive the multifunctionality of Hsp70s. Often there is ambiguity as to which class a Hsp40 protein may belong as the G/F region is ill-defined. The G/F region acts

as a linker between domains but its functional importance in Hsp70 stimulation or client protein binding has been disputed and remains unclear.

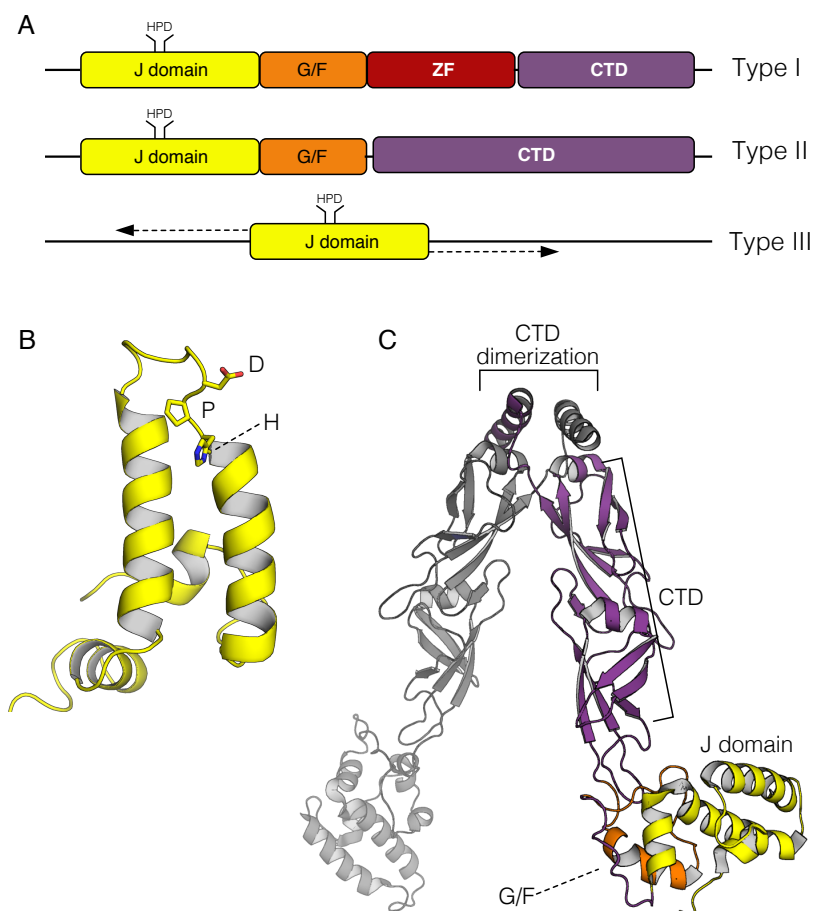


Figure 1.5: Classification of Hsp40 cochaperones. **A:** Classification of Hsp40s based on domain organisation. All three types of Hsp40, I-III, contain a J domain. Type I and II Hsp40s additionally contain a glycine and phenylalanine rich region (G/F) following the J domain and a C-terminal domain shown to bind peptides (CTD). Type I Hsp40s also contain a zinc-finger domain (ZF). Type III Hsp40s also differ from I and II by the position of its J domain; Type I and II have the J domain at the N-terminus of the protein but in type III the J domain can be anywhere in the protein. **B:** The structure of the J domain of *E. coli* DnaJ solved using NMR (PDB 1XBL) [63]. **C:** The structure of full-length dimeric *Thermus thermophilus* DnaJ (PDB 4J80), a Type II Hsp40, created by combining crystal structures with electroparamagnetic resonance (EPR) data [61].

Hsp40 proteins are able to bind and deliver client protein to the Hsp70 and stimulate ATPase hydrolysis through the J domain. Hsp40s can also function without client binding and simply act to stimulate Hsp70 action. In addition to their role in stimulating the activity of ATP hydrolysis, many Hsp40 protein have

Hsp70-independent activity and are able to prevent aggregation and modulate protein complex stability alone [49]. Many studies have attempted to elucidate the mechanism through which the J domain is able to stimulate ATP hydrolysis, but the picture is still far from complete.

NMR studies indicated that the binding site for both ADP- and ATP-bound DnaK (*E. coli* Hsp70) was located in helix II and the HPD motif of the J domain of DnaJ (*E. coli* Hsp40) [64]. This work led to the model that the HPD motif mediates docking of the negatively charged helix II onto a positively charged groove on the underside of the ATPase binding domain. Later work indicated that the J domain also interacts with a second site located on the SBD of Hsp70 and that the J domain has a greater affinity for Hsp70 when in the ATP-bound state suggesting it plays an important role in allosteric communication between domains of Hsp70 [65]. A recently solved structure of full-length DnaK in complex with the J domain of DnaJ provides us with a snapshot to understanding the mechanism of stimulation [35]. The transient nature of the complex was overcome by fusing the two proteins together by joining the G/F region of DnaJ and the N-terminus of DnaK to form a covalent linker, thereby increasing the local concentration of subunits. The complex was further stabilised by amino acid mutations to trap DnaK in its ATP-bound form. The crystal structure revealed that the J domain interacts with an interface formed between the ATPase domain, the interdomain linker and SBD β of Hsp70. The interdomain linker has inserted into a groove on the underside of the ATPase domain as in previous ATP-bound Hsp70 structures. The interaction occurs through contacts with helix II and III of the J domain with the SBD β and ATPase domain/linker respectively. The interaction of the J domain with the SBD β was shown to be necessary for the J domain – substrate synergistic mechanism that results in proper alignment of catalytic residues in the ATPase domain to achieve high rates of ATP hydrolysis.

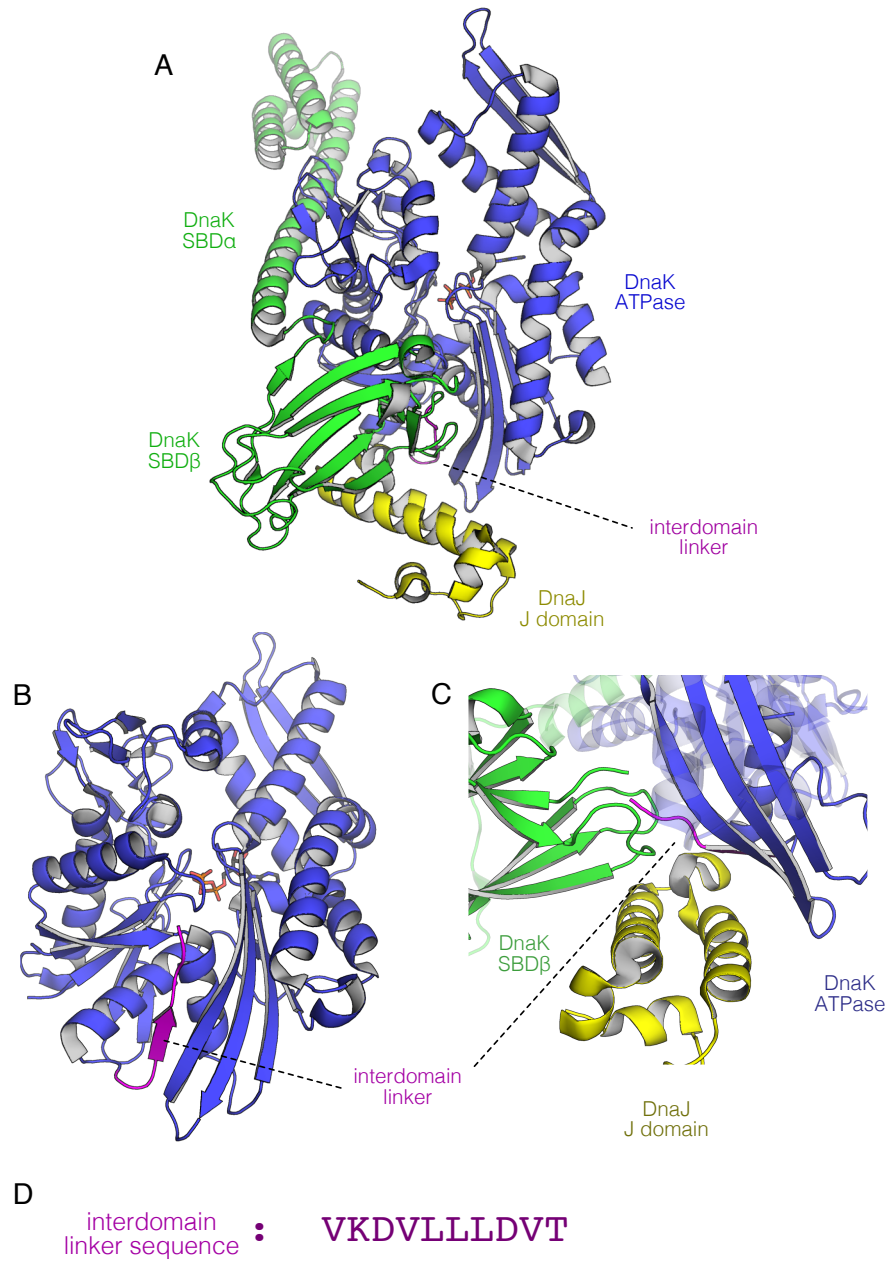


Figure 1.6: The DnaK-DnaJ complex. **A:** The recently solved crystal structure of full-length ATP-bound *E. coli* DnaK in complex with the J domain of DnaJ (PDB 5NRO) [35]. The complex structure was achieved by fusing the two partners using the G/F linker to increase local concentration. The ATPase domain of DnaK is shown in blue, the SBD in green and the interdomain linker in magenta. The J domain of DnaJ is coloured yellow. **B:** The interdomain linker (magenta) inserts into the underside of the ATPase domain (blue) allowing domain rotation causing the ATPase surface to remodel and expose areas important in SBD domain docking. **C:** A zoomed view of the interface between the DnaJ J domain and DnaK. The J domain interacts with a beta-sheet on the underside of the ATPase domain, the interdomain linker and the SBD β . **D:** The sequence of DnaK interdomain linker.

1.4 Molecular chaperones of *P. falciparum*

The *P. falciparum* parasite undergoes a great deal of stress in the red blood cell stage from fever, haemoglobin digestion and reactive oxidative species. *P. falciparum* encodes a wide range of chaperones to survive such stresses and to aid the drastic remodelling of the red blood cell necessary for pathogenesis. The parasite genome encodes at least 95 chaperones making up 2% of the genome which are expressed at different points in the parasites lifecycle and localised to different subcellular compartments [66]. Among these *P. falciparum* (Pf) chaperones are two PfHsp110 proteins, four PfHsp100/Clp proteins, an essential Hsp90 and two more putative Hsp90s, two PfHsp60s, six Hsp70-like proteins, 50 PfHsp40s and several small Hsps. In comparison, the human genome encodes 13 Hsp70s, 40 Hsp40s and 9 Hsp90s amongst other chaperones indicating the disproportionate number of PfHsp40s in the parasite.

P. falciparum Hsp70s are found in various cellular compartments and thus are thought to carry out specialized functions (Fig. 1.7) [67]. PfHsp70-1 is localised to the parasite cytosol and is one of the most highly expressed *P. falciparum* genes with expression induced further by stress such as heat shock and antimalarial drug pressure [68]. The PfHsp70-1 homologue, PfHsp70-2, localises to the ER of the parasite where it is able to channel damaged proteins to the ER associated degradation (ERAD) pathway [67]. PfHsp70-2 is found expressed in the blood stage of infection along with PfHsp70-1. PfHsp70-1 has been the main topic of malarial chaperone research to date, with numerous studies performed showing that PfHsp70-1 functions as a chaperone that is able to rescue thermally sensitive *E. coli* and is essential to the parasite's survival [67]. PfHsp70-3 is the mitochondrial Hsp70 homologue and is thought to serve as a motor to translocate preproteins into the mitochondria; however little experimental information on this chaperone exists [69, 70]. PfHsp70-y and PfHsp70-z are in fact Hsp110 homologues and are believed to serve as nucleotide exchange factors for PfHsp70-2 and PfHsp70-1 respectively due to corresponding cellular localisation [67]. It is possible that PfHsp70-y and PfHsp70-z have additional roles outside acting as nucleotide exchange factors. The

sixth PfHsp70, PfHsp70-x, is exported from the parasite into the parasitophorous vacuole and beyond to the red blood cell cytosol.

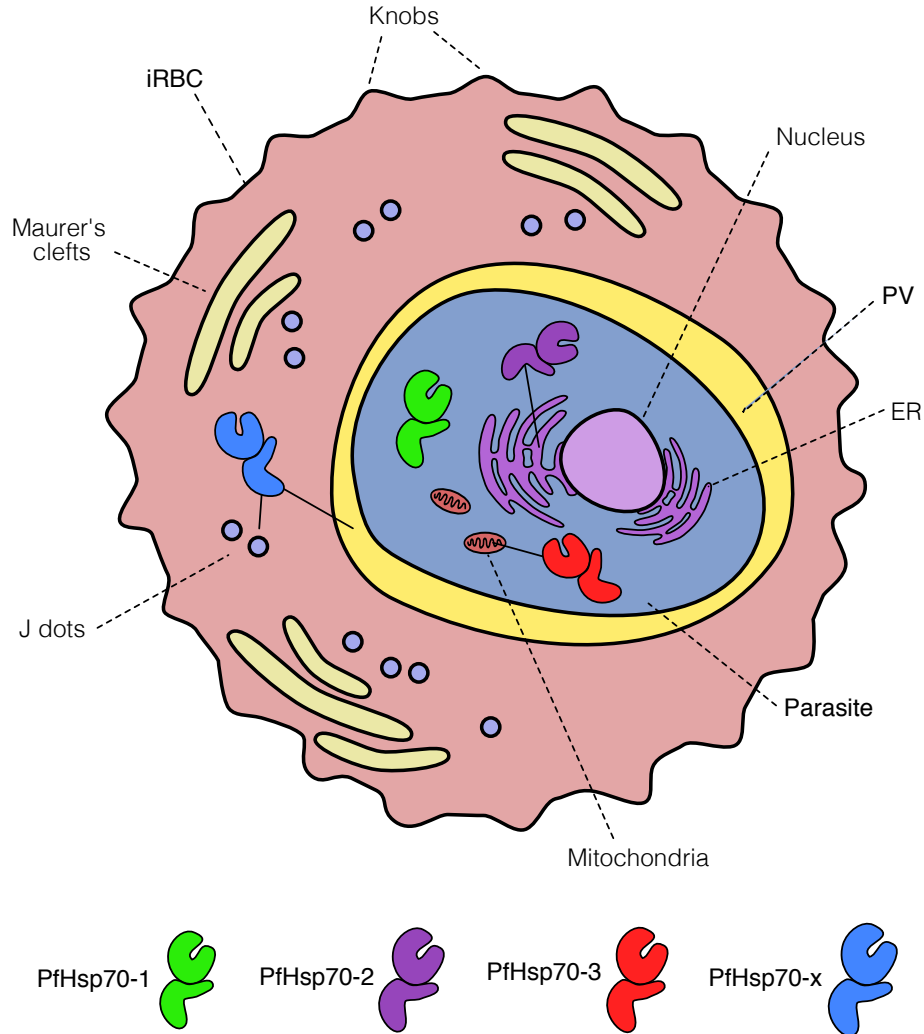


Figure 1.7: *P. falciparum* Hsp70s. A: The infected red blood cell (iRBC) after the extensive remodelling caused by *P. falciparum* infection. Remodelling of the iRBC causes the formation of cytoadherent knobs on the cell surface and the establishment of parasite induced structures such as Maurer's clefts and J dots. The parasite resides inside the parasitophorous vacuole (PV).

1.4.1 PfHsp70-x – the exported Hsp70

Reannotation of the *P. falciparum* genome lead to the discovery of the sixth Hsp70, PfHsp70-x, previously thought to be a pseudogene [71]. Hsp70-x is specific to the *Laverania* sub-genus of *Plasmodium*. The *Laverania* sub-genus parasites are the most pathogenic species of *Plasmodia* as infected erythrocytes cytoadhere to

host cell surfaces via parasite-exported virulence factors such as the erythrocytic membrane protein 1, PfEMP1 [18]. Cytoadherence is achieved by the export of multiple proteins from the parasite into the red blood cell to remodel the host cell [72, 73, 74]. The presence of Hsp70-x only in pathogenic species of *Plasmodium* suggests a role in contributing to host cell remodelling and resultant cytoadherence leading to virulence. PfHsp70-x lies in the sub-telomeric region of chromosome 8 which is highly vulnerable to chromosomal breakage [75, 76]. However, loss of the PfHsp70-x has not been reported in any parasites isolated from patients suggesting it may be playing an essential role.

PfHsp70-x is found in the parasitophorous vacuole and the cytosol of the red blood cell despite its lack of a PEXEL export amino acid sequence [71]. Within the red blood cell, PfHsp70-x is found to co-localise with the virulence factor PfEMP1 to highly mobile, cholesterol rich structures termed ‘J dots’ particularly in ring and early trophozoite stages indicating a role for PfHsp70-x in the trafficking of PfEMP1 to the cytoadherent knobs on the cell surface (Fig. 1.8). PfHsp70-x was also found to partially associate with Maurer’s clefts, but was not found in knobs.

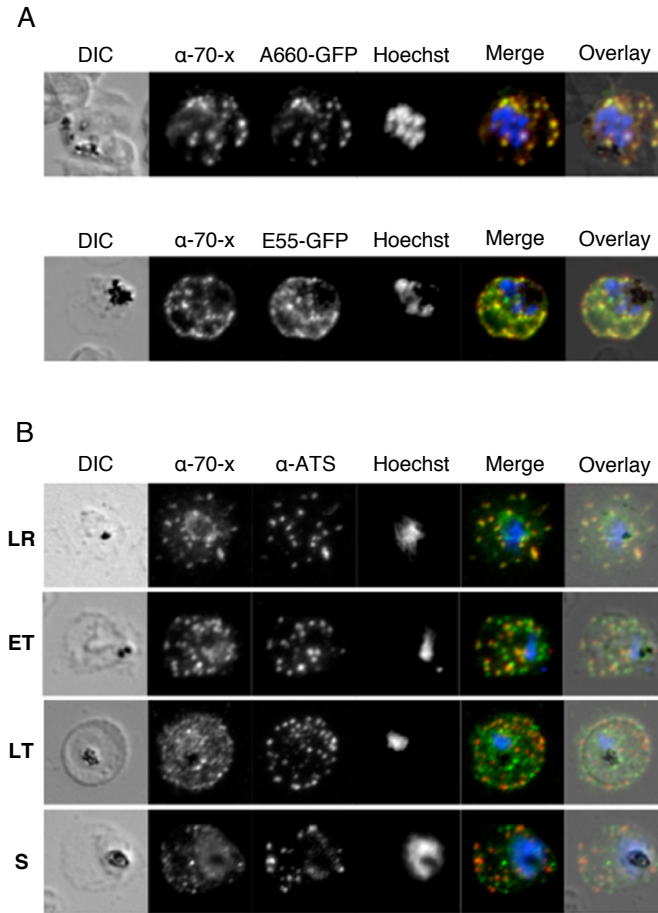


Figure 1.8: Colocalisation of PfHsp70-x with J dot Hsp40s and PfEMP1 **A:** PfHsp70-x (α -70x) is shown to colocalise with PFA0660w (A660-GFP) and PFE0055c (0055-GFP) in punctuate dots within the host cell cytosol. **B:** Localisation of PfHsp70-x through the intraerythrocytic life cycle (LR: late ring; ET: early trophozoite; LT: late trophozoite; S: schizont). In the LR and ET stages, PfHsp70-x colocalises with PfEMP1 (α -ATS). Taken from [71].

In addition to the proposed role of trafficking pathogenic parasite proteins to the surface of the host cell, a role in protein translocation across the parasitophorous vacuole membrane (PVM) into the red blood cell cytosol has been proposed. Protein translocation across the PVM occurs via a protein pore complex called PTEX and requires the exported protein to be in an unfolded state [77]. PTEX consists of five core components including a Hsp100-class chaperone, Hsp101, which has been shown to be essential for parasite viability [78]. Hsp100 proteins are able to interact with Hsp70 chaperones and it is thought that PfHsp70-x is perfectly localised in the PV and the host cell cytosol to interact with this component of PTEX, thereby

allowing the folding and unfolding of export proteins necessary for translocation.

The mature red blood cell is anuclear and thus is not able to regulate protein expression like a nucleated cell. The life span of a red blood cell is therefore relatively short (~100 days) as it cannot respond to environment changes or protein deterioration. It is thought that PfHsp70-x is exported to work in concert with the stock complement of human Hsp70 proteins present in the cell, such as Hsc70, and to provide additional stress relief to the parasite. As previously mentioned, the parasite and the infected red blood cell are exposed to much stress in the form of heat shock and oxidative stress suggesting an important role of PfHsp70-x in the survival and proliferation of the parasite in such times. It has been reported that the heat shock caused by fever actually increases proliferation of ring stage parasites and causes increased cytoadherence demonstrating that stress plays an integral part in the parasites life cycle and pathogenicity [79, 80].

PfHsp70-x is a canonical Hsp70 in that it consists of an ATPase domain and an SBD joined by a flexible, hydrophobic linker. The chaperone has been shown to have ATPase activity and folding abilities *in vitro*. PfHsp70-x lacks the PEXEL motif usually necessary for signalling export but instead has an N-terminal 25 amino acid sequence that functions as an ER-type signal sequence allowing secretion into the PV followed by a novel octameric sequence that causes export further into the host cell cytosol [71]. A number of non-PEXEL containing exported proteins (PEXEL negative exported proteins: PNEPs) have previously been reported but most contain a membrane spanning region necessary for export [77]. The novel export signal sequence of PfHsp70-x indicates an alternate pathway of export that may not involve the PTEX complex. Following the PfHsp70-x SBD, there is a stretch of 30 amino acids predicted to be disordered. At the C-terminus of the protein is an EEVN motif similar to the EEVD motif characteristic of cytosolic Hsp70 proteins which has been implicated in cochaperone binding and the allosteric mechanism of substrate folding. The EEVN motif of PfHsp70-x was shown to be important for conformational integrity and involved in binding the human Hsp organising protein (Hop), a co-chaperone that mediates the interaction of Hsp70s with Hsp90s [81].

A number of recent studies have genetically modified *P. falciparum* to explore the role of PfHsp70-x in parasite viability and pathogenicity. Work by Charnaud *et al* generated PfHsp70-x knockout parasite lines (Δ PfHsp70-x) and found that PfHsp70-x was not necessary for parasite viability and growth under standard *in vitro* culturing conditions [82]. They additionally tested the effect of stress on Δ PfHsp70-x parasites in the form of heat stress, oxidative stress, nutrient deprivation and culturing in aged erythrocytes. It was found that Δ PfHsp70-x parasites are as susceptible to most stressors as control parasites except for a slight growth defect upon oxidative stress and hypoxanthine restriction. Of particular interest, Δ PfHsp70-x parasites were shown to have a reduced virulence phenotype with less efficient export of PfEMP1 (32% reduction in PfEMP1 exposure at 26-28 hpi) and cytoadherence to the chondroitin sulfate A receptor (reduction in binding of 63%). The infected red blood cells also had increased stiffness of the membrane causing increased cell rigidity, which *in vivo* might result in increased iRBC clearance by the spleen. thus, this study confirmed the role of PfHsp70-x in the export and/or trafficking of parasitic proteins involved in pathogenesis. It was found, however, that Δ PfHsp70-x parasites had increased expression of several exported proteins such as the kinase FIKK13 and PHIST proteins including PFE1605w and the ring-infected erythrocyte surface antigen (RESA). Additionally, PfHsp70-3, the mitochondrial PfHsp70, was found to be upregulated. At the same time, numerous proteins were found to be downregulated, including many exported proteins of unknown function and the intraparasitic Hsp110. Charnaud *et al* suggested that the change in gene expression may be due to selection pressure to compensate for the loss of PfHsp70-x, thereby masking some effects of PfHsp70-x loss. A seperate study by Cobb *et al* generated a post-transcriptional conditional knockdown of PfHsp70-x capable of rapidly reducing PfHsp70-x levels without allowing selection of compensating parasites to occur [83]. This study found that the knockdown cell line showed no significant effect on growth or viability under standard culturing conditions but did not explore the effect of stress on the knockdown line.

A recent study performed by Mabate *et al* [81] aimed to further characterise PfHsp70-x *in vitro* found this chaperone had a preference for binding arginine-rich peptides. Arginine-repeat rich proteins make up over 25% of the *P. falciparum* proteome and are known to be prone to aggregation therefore it seems likely that PfHsp70-x preferentially binds such proteins compared to human Hsp70s.

Work towards inhibiting PfHsp70-x and other PfHsp70s was carried out by Cockburn *et al*. Initially, a screen for small molecules that modulate the protein aggregation suppression activity of PfHsp70-1 was performed [84]. A number of compounds were identified that also possessed antimalarial activity with certain small molecules showing preferential inhibition of PfHsp70-1 compared to human Hsp72. Further work by Cockburn *et al* [85] concentrated on this new class of antimalarials, testing their effect on the ATP hydrolysis activity of PfHsp70-1 and PfHsp70-x in comparison to the human Hsp72 to determine specificity. Two compounds were found to be particularly interesting in their modulatory effects: malonganenone A (MA) and bromo-beta-lapachol (BBL). MA was not capable of inhibiting the basal ATPase activity of any of the Hsp70 proteins but was able to inhibit the stimulated activity of certain Hsp70/Hsp40 pairs – particularly PfHsp70-1/PfHsp40. BBL strongly inhibited the ATPase activity of PfHsp70-x (reduced to 20 % of control) but not of other Hsp70s tested. BBL was also capable of inhibiting the Hsp40-stimulated activity of PfHsp70-x and PfHsp70-1 depending on the Hsp40 used. Both compounds had antimalarial activity with IC₅₀ values of 18 μ M and 800 nM for BBL and MA respectively. BBL showed to be highly cytotoxic to mammalian cells but MA showed minimal cytotoxicity. These studies have suggested that preferential inhibition of PfHsp70-x activity by small molecules is possible despite the high conservation of Hsp70 proteins, and also showed the potential for targeting specific Hsp70-Hsp40 partners. Targeting Hsp70-Hsp40 complexes is proposed to be particularly promising in developing modulators of Hsp70 activity, as there is a greater specificity to these interactions which allows the fine-tuning of the chaperone network [86].

1.4.2 J dot resident PfHsp40s

PfHsp70-x is found incorporated into high molecular weight complexes in the infected red blood cell alongside Hsp40s of parasitic origin [87]. The complexes are found in J dots which are highly mobile parasite induced structures and named after the presence of Hsp40s also known as J proteins [88]. Two type II parasite Hsp40s, PFE0055c and PFA0660w, are exported into the host cell cytosol where they colocalise with J dots (Fig. 1.8A). It was found that the two Hsp40 proteins are tightly associated with J dots despite lacking a hydrophobic membrane spanning region and it has been shown that localisation to these cholesterol-rich structures relies on the C-terminal substrate binding region of the Hsp40s [88, 89]. Attempts to create a PFA0660w knockout line have so far been unsuccessful implying that this Hsp40 is essential, whereas PFE0055c has been successfully knocked-out [90].

Daniyan *et al* [91] demonstrated that both PFA0660w and PFE0055c are capable of stimulating the chaperone activity of PfHsp70-x *in vitro* providing evidence for functional Hsp70/Hsp40 complexes in J dots. In particular, they found that PFA0660w specifically stimulates the activity of PfHsp70-x but not the human Hsp72. PFA0660w was also shown to have considerable aggregation suppression activity alone showing the ability of the Hsp40 to bind substrates. This information suggests that PFA0660w is able to direct PfHsp70-x function by bringing specific substrates through its own substrate binding domain to be folded or trafficked.

1.5 Molecular chaperones as drug targets

Molecular chaperones have emerged as potential drug candidates due to their involvement in a number of diseases ranging from cancer, neurodegenerative diseases and infectious diseases [92]. Hsp90 proteins, perhaps the most studied class of all the chaperones, have been shown to be important for the development and proliferation of several types of cancer and thus Hsp90 inhibitors such as 17-allylamino-17-demethoxygeldanamycin (17-AAG), Tanespimycin and Ganetespide have been developed as anti-cancer drugs [93, 94, 95, 96]. Hsp60 proteins have

been implicated in inflammation and transplant rejection alongside cancer and neurodegeneration leading to the development of inhibitors such as mizoribine [97]. In animal models of Parkinson's disease, Alzheimer's disease and Huntington's disease, overexpression of Hsp70 proteins delayed disease protection leading to the development of dihydropyrimidines that can modulate the activity of Hsp40-stimulated Hsp70s [31]. Hsp70s have also been implicated in cancer as high Hsp70 levels are associated with adverse outcomes in breast, endometrial and prostate cancers as well as certain leukemias. Thus, a broad range of anti-cancer Hsp70 inhibitors have been developed in the form of ATP mimetics, spargualin-like compounds, fatty acids, peptides and various dyes.

1.5.1 Malarial chaperones - an emerging therapeutic target

Repurposing of known chaperone inhibitors as antimalarials may prove to be an effective strategy when combined with other therapeutics [98]. For example, geldanamycin (GA), an Hsp90 inhibitor and precursor of 17-AAG, can act synergistically with chloroquine to efficiently kill parasites *in vitro* [99]. GA also proved to be effective against chloroquine-resistant parasites suggesting that the combination of these two drugs could be an effective option.

The exported malarial chaperones, PfHsp70-x and associated PfHsp40s, make particularly interesting targets as small compounds targeting the erythrocyte cytoplasm have fewer membranes to transverse; inhibition of intraparasitic chaperones require small compounds to cross the parasite membrane and the parasitophorous vacuole membrane in addition to the host cell membrane. However, as Hsp70 chaperones play such diverse roles in the cell and fold hundreds of proteins, broad-spectrum inhibition of Hsp70s could lead to adverse and unpredicted effects. Therefore, we must aim to specifically inhibit PfHsp70-x, which is potentially difficult as Hsp70s are highly conserved, or aim to break interactions between PfHsp70-x and its cochaperones. Targeting Hsp70-Hsp40 complexes, such as PfHsp70x – PFA0660w, has been suggested to be particularly attractive as one can aim to disrupt specific chaperone-cochaperone pairs and fine-tune chaperone network.

1.6 Aims and work presented

In this thesis, I aim to address the following questions:

1. Does PfHsp70-x play a role in protecting the parasite from stress?
 - (a) Does this make PfHsp70-x a suitable drug target?
2. Does PfHsp70-x differ from human Hsp70s in structure or function?
 - (a) Can we specifically inhibit PfHsp70-x?
 - (b) Can we gain insights on its role?
3. Can we inhibit PfHsp70-x towards repurposing existing Hsp70 inhibitors as antimalarials?
4. Can the PfHsp70-x - Hsp40 interaction be specifically targeted?

Chapter 3 probes the role of the exported parasite Hsp70, PfHsp70-x, in response to stress using genetically modified *P. falciparum* parasites and thus attempts to answer Question 1. The following chapter aims to structurally characterise the catalytic domain of PfHsp70-x allowing comparison to human Hsp70s to answer Question 2. Question 3 is explored in Chapter 5 as we investigate the inhibition of PfHsp70-x to test the potential of repurposing Hsp70 inhibitors as antimalarials and to understand so-called specific inhibitors of the malarial chaperone. Finally, Chapter 6 investigates the interactions of PfHsp70-x with J dot resident Hsp40s, PFA0660w and PFE0055c, to understand the preferential stimulation of chaperone activity by these proteins towards answering Question 4. Taken together, these results will enable the future design of small molecules that can modulate and fine-tune the chaperone network in the malaria infected erythrocyte to reduce pathogenesis and parasite proliferation. Chapter 7 discusses the impact of work presented here and the future directions of studying the chaperone network of the *P. falciparum* infected erythrocyte.

2

Methods

Contents

2.1	Cloning	29
2.2	Protein Expression and Purification	30
2.2.1	PfHsp70-x, Hsc70, PFA0660w _J , PFE0055c _J	30
2.2.2	DnaJ	33
2.3	NMR	34
2.3.1	HSQC Titrations	34
2.3.2	Backbone Assignments	35
2.3.3	Saturation Transfer Difference	35
2.4	Biophysics and Biochemical Assays	36
2.4.1	Mass Spectrometry	36
2.4.2	Circular Dichroism	36
2.4.3	MESG ATP Hydrolysis Assay	37
2.5	X-Ray Crystallography	37
2.5.1	Structure Determination of PfHsp70-x _{ATPase} :AMP-PNP	37
2.5.2	Structure Determination of PfHsp70-x _{ATPase} :ADP	38
2.5.3	Inhibitor Soaking of PfHp70-x _{ATPase} Crystals	38
2.5.4	Structure Determination of PFA0660w _J	39
2.6	Computational methods	39
2.6.1	Homology Modelling	39
2.7	<i>In vitro Plasmodium falciparum</i> Assays	40
2.7.1	Parasite Culturing	40
2.7.2	PfHsp70-x knockdown: induction and validation	41
2.7.3	Stress Assays	41
2.7.4	Drug Assays	43
2.8	Graphs and Structures	43

2.1 Cloning

Plasmodium falciparum PfHsp70-x (Uniprot accession number K7NTP5) constructs were derived from a codon optimised synthetic DNA fragment from Integrated DNA Technologies (IDT) encoding for protein residues 25 – 679. Human Full length Hsc70 (Uniprot accession number P11142) was cloned from sequence verified cDNA from the Source BioScience I.M.A.G.E Consortium. *Plasmodium falciparum* PFA0660w (Uniprot accession number Q8I2E1) and PFE0055c (Uniprot accession number Q8I489) constructs were generated using codon optimised synthetic DNA fragments from IDT encoding for protein residues 81 – 402 and 77 – 402 respectively. DnaJ (Uniprot accession number P08622) was amplified from *Escherichia coli* strain Rosetta2(DE3) genomic DNA obtained from cell lysate.

PCR was used to amplify DNA sections using primer oligos from Sigma. Amplified regions were cloned into a pFloat vector containing an N-terminal His6-tag and a human rhinovirus (HRV) 3C protease cleave site [100] using Gibson Assembly (New England BioLabs). Resulting assembly products were transformed into 5-alpha competent *E. coli* cells (New England BioLabs). Transformed cells were plated onto 1.5 % w/v Luria Bertani (LB) agar plates containing 50 $\mu\text{g mL}^{-1}$ kanamycin for antibiotic selection of correctly transformed cells. Plates were incubated for 16 hours at 37 °C. Colonies were picked and used to inoculate separate 10 mL LB cultures with 50 $\mu\text{g mL}^{-1}$ kanamycin. LB cultures were grown overnight for 16 hours at 37 °C . Cells were harvested, and plasmid DNA extracted using the Qiagen Miniprep kit according to manufacturer's instructions. Plasmid DNA was sequenced by Eurofins Genomics to confirm correct cloning of constructs.

Table 2.1: Table of constructs.

Protein name (UniProt accession number)	Construct name	Construct boundaries
PfHsp70-x (K7NTP5)	PfHsp70-x _{FL}	25 - 679
	PfHsp70-x _{ATPase}	29 - 419
Hsc70/HSPA8 (P11142)	Hsc70 _{FL}	1 - 638
PFA0660w (Q8I2E1)	PFA0660w _J	81 - 157
PFE0055c (Q8I489)	PFE0055c _J	77 - 148
DnaJ (P08622)	DnaJ	1 - 376

2.2 Protein Expression and Purification

2.2.1 PfHsp70-x, Hsc70, PFA0660w_J, PFE0055c_J

DNA constructs were transformed into *E. coli* strain Rosetta2(DE3) cells for protein expression and grown in LB or, for NMR usage, in M9 minimal media supplemented with ^{15}N NH_4Cl and/or $^{13}\text{C}_6\text{D}$ -glucose as appropriate at 37°C with $50\text{ }\mu\text{g mL}^{-1}$ kanamycin and $34\text{ }\mu\text{g mL}^{-1}$ chloramphenicol. Upon reaching an OD_{600} of 0.5-0.6, the incubation temperature was reduced to 18°C and protein expression initiated by addition of isopropyl β -D-1-thiogalactopyranoside (IPTG) to a final concentration of $250\text{ }\mu\text{M}$. Protein expression was allowed to proceed for 18 hours before harvesting.

Cells were harvested by centrifugation and resuspended in ATP lysis buffer (10 mM Na_2HPO_4 , 2 mM KH_2PO_4 , 2.7 mM KCl, 500 mM NaCl, 0.01% Triton X-100, 5 mM ATP, 2 mM MgCl_2 pH 7.4) in the case of PfHsp70-x and Hsc70 proteins or lysis buffer (10 mM Na_2HPO_4 , 2 mM KH_2PO_4 , 2.7 mM KCl, 500 mM NaCl, 0.01% v/v Triton X-100 pH 7.4) for PFA0660w and PFE0055c J domains. Sonication was used to lyse resuspended cells and the resulting lysate was clarified by centrifugation at $30,000\times g$. The clarified lysate was loaded onto a pre-equilibrated HiTrap TALON metal affinity column (GE Healthcare Life

Sciences) and washed with the appropriate lysis buffer. Proteins were eluted with lysis buffer supplemented with 500 mM imidazole.

Proteins were extensively dialysed in 3C cleavage buffer (20 mM Na₂HPO₄, 150 mM NaCl, 1 mM 1,4-Dithiothreitol (DTT) pH 7.4) and incubated with recombinant HRV 3C protease to cleave the purification tag at 4 °C for 16 hours [101]. Size exclusion chromatography was performed as the final purification step using Superdex 75 or Superdex 200 columns (GE Healthcare Life Sciences) equilibrated in NMR buffer (20 mM Na₂HPO₄, 150mM NaCl, 2 mM DTT pH 7.4), crystallography buffer (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 50 mM NaCl, 1 mM DTT pH7.4) or assay buffer (10 mM HEPES, 100 mM NaCl, 0.5 mM DTT, 2 mM MgCl₂ pH 8).

Protein purity was assessed by sodium dodecyl sulphate-polyacrylamide electrophoresis (SDS-PAGE) (2.1 and 2.2). Proteins were concentrated using spin ultrafiltration with concentrations calculated by UV absorption at 280 nm and an extinction coefficient estimated from amino acid sequence [102].

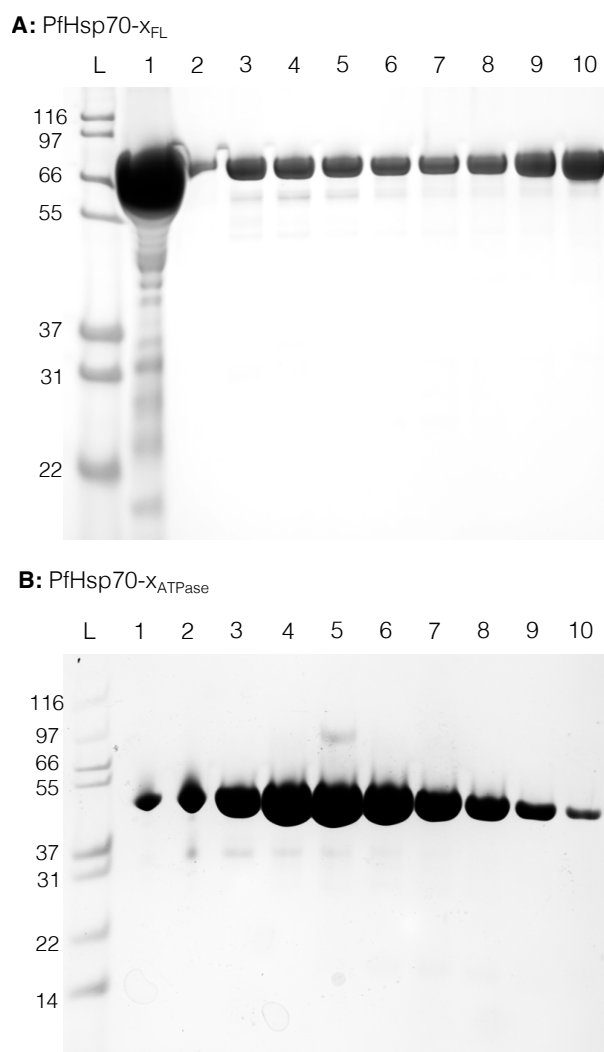


Figure 2.1: SDS PAGE of purified PfHsp70-x FL and ATPase constructs.
A: SDS PAGE of PfHsp70-x_{FL} after size exclusion. L = Mark12 Unstained Ladder (ThermoFisher Scientific). 1 indicates the post affinity chromatography sample. 2-10 are samples from size exclusion. 2-10 all have purity of >95% with 6-10 having a purity of >99% as determined by ImageJ. **B:** SDS PAGE of PfHsp70-x_{ATPase} after size exclusion. L = Mark12 Unstained Ladder (ThermoFisher Scientific). 1-10 are samples after size exclusion. 1-10 all have purity of >95% with 1,4 and 6-10 having a purity of >99% as determined with ImageJ.

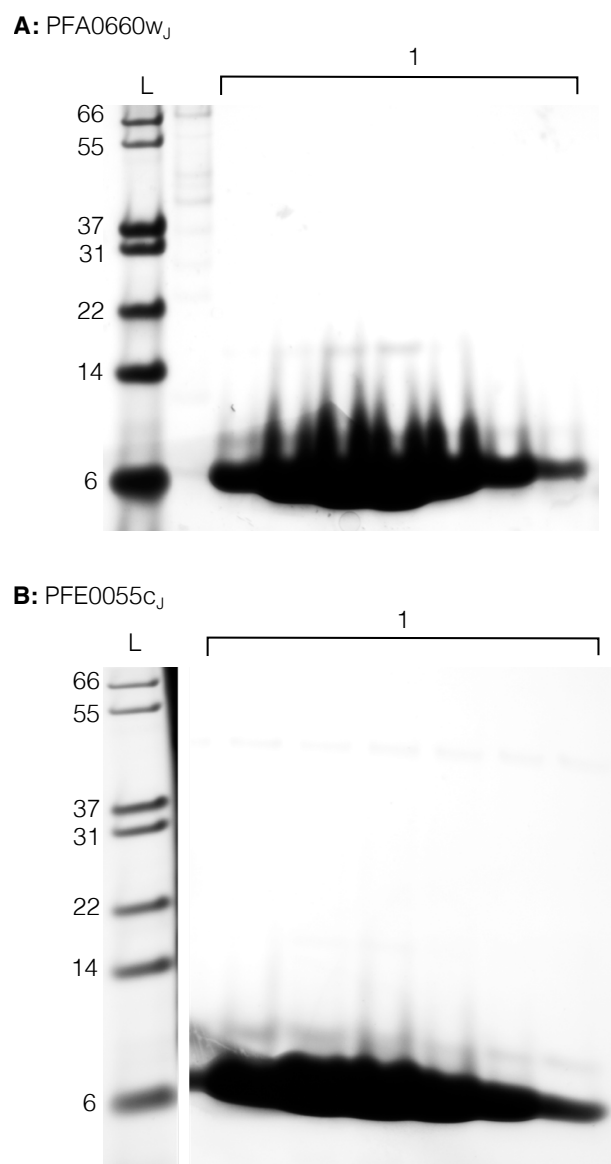


Figure 2.2: SDS PAGE of purified PFA0660w and PFE0055c J domains. **A:** SDS PAGE of PFA0660w_J after size exclusion. L = Mark12 Unstained Ladder (ThermoFisher Scientific). 1 indicates the post size exclusion samples with a purity of >95% as determined by ImageJ. **B:** SDS PAGE of PFE0055c_J after size exclusion. L = Mark12 Unstained Ladder (ThermoFisher Scientific). 1 indicates the post size exclusion samples with a purity of >99% as determined by ImageJ.

2.2.2 DnaJ

The DnaJ full length construct was transformed into *E. coli* Rosetta2(DE3) for protein expression and grown in LB at 37°C. Upon reaching an OD₆₀₀ of 0.5-0.6, protein expression was initiated by addition of IPTG to a final concentration of 250

μ M and protein expression was allowed to proceed for 5 hours before harvesting.

Cells were harvested by centrifugation and resuspended in mild denaturant lysis buffer (10 mM Na_2HPO_4 , 2 mM KH_2PO_4 , 2.7 mM KCl, 500 mM NaCl, 0.01% v/v Triton X-100, 2 M Urea, pH 7.4). Sonication was used to lyse resuspended cells and the resulting lysate was clarified by centrifugation at 30,000 x g. The clarified lysate was loaded onto a pre-equilibrated HiTrap TALON metal affinity column and washed with lysis buffer. Proteins were eluted with mild denaturant lysis buffer supplemented with 500 mM imidazole.

Proteins were then dialysed in 2-(N-morpholino)ethanesulfonic acid (MES) dialysis buffer (50 mM MES, 50 mM NaCl, 1 mM DTT, 2 mM EDTA pH 6) and incubated with HRV 3C protease to cleave the purification tag.

Ion exchange chromatography using a HiTrap SP HP Sepharose cation exchange column (GE Healthcare Life Sciences) was used as the final purification step in which proteins were eluted by a gradient of MES dialysis buffer supplemented with 1 M NaCl. Protein was then dialysed into assay buffer (10 mM HEPES, 100 mM NaCl, 0.5 mM DTT, 2 mM MgCl_2 pH 8).

Protein purity was assessed at each step by SDS-PAGE. Protein was concentrated using spin ultrafiltration with concentrations calculated by UV absorption at 280nm.

2.3 NMR

All NMR experiments were conducted using Bruker Avance III spectrometers with cryogenic TCI probeheads operating at field strengths of 500, 600 or 750 MHz and controlled with Bruker TopSpin software. Samples consisted of protein in NMR buffer supplemented with 5% w/v D_2O and 50 μ M 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) unless stated otherwise. Data were processed with NMRpipe [103] and analysed with Sparky [104] or TopSpin software.

2.3.1 HSQC Titrations

Heteronuclear single quantum coherence (HSQC) NMR spectra were recorded with samples of ^{15}N isotopically enriched protein at 100 μ M concentration unless

stated otherwise. Spectra of PfHsp70-x_{ATPase} and PFA0660w_J and PFE0055c_J were collected at 25 °C. Spectra were collected with increasing amounts of either unlabelled protein or small molecule and overlaid for comparison and analysis. Where percentage change of peak intensity is discussed, the following equation was used to calculate this value:

$$PC = \frac{PI_{\text{complex}} - PI_{\text{apo}}}{PI_{\text{apo}}} \times 100 \quad (2.1)$$

Where, PC is percentage change in intensity, PI_{apo} is the peak intensity of the residue with only labelled protein and PI_{complex} is the peak intensity of the residue with labelled protein and ligand/binding partner. Associated errors were calculated from the estimated level of noise for each spectrum using the following equation, derived from the propagation of uncertainties principle, to propagate errors through the percentage change calculation [105].

$$\frac{\delta PC}{PC} = \sqrt{\left(\frac{\sqrt{(\delta PI_{\text{complex}})^2 + (\delta PI_{\text{apo}})^2}}{PI_{\text{complex}} - PI_{\text{apo}}} \right)^2 + \left(\frac{\delta PI_{\text{apo}}}{PI_{\text{apo}}} \right)^2} \quad (2.2)$$

2.3.2 Backbone Assignments

Sequential resonance assignments of the backbones of PFA0660w_J and PFE0055c_J were performed using 3D HN(CO)CACB and HNCACB experiments at 25 °C and protein sample concentrations at 1 mM [106, 107].

2.3.3 Saturation Transfer Difference

Saturation transfer difference (STD) experiments were used to probe small molecule binding to proteins. Proteins were buffer exchanged into D₂O PBS (10 mM Na₂HPO₄, 2.7 mM KCl, 137 mM NaCl pH 7.4 in 100 % v/v D₂O) using SpinTrap 25 columns (GE Healthcare Life Sciences) to minimise ¹H signal from water. Methylene blue and BBL were dissolved in D₂O and deuterated dimethyl sulfoxide (DMSO) respectively. Irradiation frequencies for data collection were optimised at the maximum small compound concentration of 1 mM with 10 μM protein. Both

on- and off-resonance frequencies were irradiated to allow calculation of the STD amplification factor. Data were collected at decreasing concentrations of small molecules and also with small molecules alone as controls. All STD experiments were performed at 10 °C. The STD amplification factor for each ^1H resonance was calculated using the equation below [108]:

$$STD_{\text{amp of resonance}} = \frac{STD_{\text{diff}}}{STD_{\text{off}}} \times \text{ligand excess} \quad (2.3)$$

Where STD_{amp} is the STD amplification factor, STD_{diff} is the ^1H resonance intensity of the STD difference spectrum and STD_{off} is the ^1H resonance intensity of the off-resonance spectrum.

STD experiments were used to estimate the dissociation constant, K_D , of binding using the equation below [108]:

$$STD_{\text{amp}} = \frac{\alpha_{\text{STD}}[L]}{K_D + [L]} \quad (2.4)$$

Where α_{STD} is the maximum amplification factor and L is the ligand concentration.

2.4 Biophysics and Biochemical Assays

2.4.1 Mass Spectrometry

Protein identity and mass were confirmed with time of flight mass spectrometry using a Micromass LCT.

2.4.2 Circular Dichroism

Circular dichroism (CD) was used to confirm correct folding, secondary structure composition and thermal stability of proteins. All spectra were measured using a Jasco J-815 Spectropolarimeter in a quartz cuvette with a 1 mm path length with samples in diluted NMR buffer. Initial spectra were recorded between 260 – 180 nm at 20 °C and buffer-only background subtracted. Thermal stability was measured by following the CD signal at 222 nm at temperatures increased from 10 – 80 °C at a rate

of 1 °C/min. Spectra were also collected at 15 °C following thermal denaturation to the check ability of the protein to refold. Data were normalised for sample concentration, number of amino acids and the molecular weight of the protein.

2.4.3 MESG ATP Hydrolysis Assay

The rate of ATP hydrolysis was measured using a coupled enzymatic reaction in which purine nucleoside phosphorylase enzyme (PNPase) uses released inorganic phosphate (Pi) to modify the substrate 2-amino-6-mercapto-7-methylpurine riboside (MESG) causing absorption at 360 nm to increase [109]. All MESG assays were performed using a CLARIOstar microplate reader (BMG LABTECH). 5 μ M chaperone was incubated with 400 μ M MESG and 2 U PNPase in assay buffer for 30 minutes at 25 °C before the addition of ATP to a final concentration of 1 mM. The absorbance at 360 nm was followed over 2 hours and the steady state reaction rate was calculated. Values were converted to moles of ATP hydrolysed using a standard curve derived from variable concentrations of inorganic phosphate samples.

2.5 X-Ray Crystallography

For all crystallization trials, optimal concentration of protein was determined using pre-crystallisation tests (Hampton Research). Crystallization trials involved a broad approach using commercial screens from Qiagen (JCSG I – IV) and Molecular Dimensions (Morpheus). Screens were set up in sitting drop vapour diffusion plates (TTP Labtech) using a Mosquito robot to deposit 200 nL drops at 1:1 and 2:3 ratios of protein to mother liquor in each plate well. Commercial screens were set up at both room temperature and 4 °C to increase the screening space.

2.5.1 Structure Determination of PfHsp70-x_{ATPase}:AMP-PNP

Diffracting crystals from a sample of 15 mg mL⁻¹ PfHsp70-x_{ATPase} in complex with adenylyl-imidodiphosphate (AMP-PNP) at a 1:10 molar ratio were obtained at 20 °C in a condition containing 24% w/v PEG 1500 and 20% v/v glycerol.

Diffraction data for these crystals were collected at Diamond Light Source beamline I03 at 100 K to a resolution of 1.81 Å. The space group was determined to be $P 2_1 2_1 2_1$ with two copies of PfHsp70-x_{ATPase} per asymmetric unit. Data were processed with XIA2 [110] and solved by molecular replacement using the automated mode in Phaser [111] with the human Hsp72 ATPase domain structure [112] (PDB 5MKR). Coot was used for model building alongside iterative refinement with Phenix Refine [113, 114]. Not all loop residues could be built due to poor electron density; chain A 215-221 residues, and chain B residues 215 – 221, and 279-283, 413 – 416. Additionally, the first 11 amino acids of both molecules could not be modelled due to missing electron density. The structural model of PfHsp70-x_{ATPase} was validated using MolProbity [115]. Statistics of data collection and refinement are shown in Table 4.1 in Chapter 4.

2.5.2 Structure Determination of PfHsp70-x_{ATPase}:ADP

Diffraction crystals for a sample of 15 mg mL⁻¹ PfHsp70-x_{ATPase} in complex with adenosine 5'-diphosphate (ADP) at a 1:10 molar ratio were obtained at 20 °C in a condition containing 0.1 M MES, 5% w/v PEG 3000, 30% v/v PEG 200 pH 6.

Diffraction data were collected at Diamond Light Source beamline I04 at 100 K to a resolution of 1.87 Å. The space group was determined to be $P 2_1 2_1 2_1$ with two molecules per asymmetric unit similar to PfHsp70-x_{ATPase}:AMP-PNP. Data were processed with XIA2 and solved using Phaser and the PfHsp70-x_{ATPase}:AMP-PNP structure without ligand as the molecular replacement search model. Coot was used for model building alongside iterative refinement with Phenix refine. The structural model was validated using MolProbity. Statistics of data collection and refinement are shown in Table 4.2 in Chapter 4.

2.5.3 Inhibitor Soaking of PfHp70-x_{ATPase} Crystals

Crystals of PfHsp70-x_{ATPase} with AMP-PNP were grown as described above. 100 mM stock solutions of compounds were made in DMSO. Crystals were harvested and then soaked in drops consisting of mother liquor and final concentrations of

either 10 mM or 1 mM of compound. Crystal soaking was allowed to proceed for 16 hours at 20 °C before being harvested and flash cooled.

Diffraction data were collected at the European Synchrotron Radiation Facility using the automated MASSIF-1 beamline at 100 K. Data were processed with automatic processing pipelines: XIA2, XDSAPP [116], autoPROC [117] and EDNA [118].

The structure of PfHsp70-x_{ATPase} without ligand was rigid body refined in Phenix using the resulting MTZ maps from crystal soaking experiments to assess structural changes, presence of ligand or modifications.

2.5.4 Structure Determination of PFA0660w_J

Crystals of PFA0660w_J from protein samples at 8.75 mg mL⁻¹ concentration were obtained at 4 °C in a condition containing a 0.12 M mix of ethylene glycol, Tris (base)/Bicine 0.1 M pH 8.5, PEGMME550 30% w/v and PEG 20,000 30% w/v.

Diffraction data were collected at Diamond Light Source beamline I24 at 100 K to a resolution of 1.39 Å. The space group was determined to be P 1 2₁ 1 with two molecules per asymmetric unit. Data were processed with autoPROC and solved by molecular replacement using Phaser and the J domain of Sis1 protein from *Saccharomyces cerevisiae* (PDB 4RWU)[119]. Coot was used for model building alongside iterative refinement with Phenix refine. The structural model of PFA0660w_J was validated using MolProbity.

2.6 Computational methods

2.6.1 Homology Modelling

A homology model of the PFE0055c_J structure was generated using the one-to-one threading mode on SWISS-MODEL [120] using the crystal structure of PFA0660w_J as the template. Secondary structure features of the resulting model were cross checked against secondary structure prediction from NMR ¹³C backbone chemical shifts performed by PECAN [121].

2.7 *In vitro Plasmodium falciparum* Assays

Plasmodium falciparum parasite lines PfHsp70-x glmS, M9 and Δ PfHsp70-x were kindly gifted by Dr. Vasant Muralidharan from University of Georgia, USA [83].

2.7.1 Parasite Culturing

Plasmodium falciparum 3D7 cell lines were cultured according to standard procedures. Briefly, parasites were cultured in Roswell Park Memorial Institute (RPMI) + Albumax complete medium (1 x RPMI 1640, 0.5% Albumax II, 25 mM HEPES, 25 mM NaHCO₃, 0.5 mL L⁻¹ gentamicin, 0.36 mM hypoxanthine, 100 μ g mL⁻¹ Neomycin). Human erythrocytes (Zurich blood bank) were used as host cells and hematocrit was maintained at 5%. Cultures were grown in modular chambers at 37 °C in culturing gas (3% O₂, 5% CO₂ and 92% N₂ by volume). Parasitaemia was measured using Giemsa smears and kept between 0.5% and 5%.

Where indicated, parasite cultures were synchronised through either ring selection or late-stage enrichment. Ring selection was performed using a sorbitol treatment [122] in which mature parasites (>12 hpi) exhibit higher sensitivity to sorbitol and are therefore lysed but ring stage parasites remain intact and viable. For sorbitol treatment, high parasitaemia cultures were centrifuged at 600 x g to form a pellet. The pellet was resuspended in 5% w/v sorbitol and incubated for 5 minutes at 37 °C. The culture was then centrifuged again at 600 x g. The resulting pellet was washed 3 times in fresh culturing medium before being diluted to 5% hematocrit and subcultured as usual.

Enrichment of late-stage infected erythrocytes was achieved using a 70% Percoll cushion made in 1 x RPMI 1640 and 3% w/v sorbitol in PBS. High parasitaemia cultures were centrifuged at 600 x g. The resuspended pellet was layered on top of the 70% w/v Percoll cushion. The layered sample was centrifuged at 1,500 x g. The upper most layer interphase consisted of trophozoites and schizonts which were washed in fresh culturing medium and subcultured as usual.

2.7.2 PfHsp70-x knockdown: induction and validation

Knockdown and control lines PfHsp70-x glmS and M9 were treated with 2.5 mM glucosamine for 48 hours before experiments were conducted [123]. Successful knockdown of PfHsp70-x was confirmed by Western blot.

PfHsp70-x knockdown Western blot

500 μ L of trophozoite-infected RBC were lysed in eight pellet volumes of 0.03 % saponin/PBS on ice for 10 minutes. The pellet was washed in PBS supplemented with Complete Proteinase Inhibitor (Roche) the washed pellet was taken up in 200 μ L Lämmli-buffer supplemented with Complete Protease Inhibitor and DNase I (Sigma). The samples were sonicated for 5 minutes at 30 second intervals and stored at -20°C until further use. Parasite lysates were thawed and boiled at 95°C for 5 minutes, vortexed and centrifuged at full speed for 1 minutes. 10 μ L of supernatant were loaded onto a NOVEX NuPAGE Bis-Tris gel (Life Technologies). Transfer to a membrane was performed using the iBlot 2 (Life Technologies). Membranes were blocked in 5 % milk in TNT (100 mM Tris, 150 mM NaCl, 100 mM fuming HCl, and 0.1 % Tween 20) and subsequently probed with primary antibodies diluted in 5 % milk/PBS. Rabbit anti-Hsp70-x (1:5,000; [71]), rat anti-HA (1:1,000; Roche), mouse anti-GAPDH (1:10,000; C. Daubenberger) were used as primary antibodies and goat anti-mouse-HRP (1:10,000; Pierce), goat anti-rabbit-HRP (1:10,000; Jackson Immunology), and goat anti-rat-HRP (1:10,000; Southern Biotech) were used as secondary antibodies. Detection was performed using chemiluminescence (SuperSignal West Pice, Thermo Scientific).

2.7.3 Stress Assays

Two types of stress were tested for effects on knockout and knockdowns of PfHsp70-x: heat stress and oxygen stress. Parasitaemia was measured using flow cytometry on a Becton Dickinson FACSCalibur system with CellQuest Pro V5.2.1 software. Infected erythrocytes were stained using SYBR Green (Sigma) diluted 1:5,000 in FACS Flow (Becton Dickinson) and incubated in the dark at 37°C for 15 minutes.

Cells were spun down and the pellet washed in FACS Flow. 100,000 events were measured for each reading. Red blood cells were gated based on scattering of light and SYBR Green binding to DNA allowed further gating of infected erythrocytes by fluorescence (Fig. 3.11).

For the asynchronous heat stress assay, parasite cultures were distributed into 6 well plates and diluted with a target parasitaemia of 0.5%. Parasitaemia was measured for each well and this was designated Time = 0. Heat stress was applied to half of the cultures in the form of incubation at 41 °C for 12 hours to imitate a febrile episode. The other half of the cultures were grown at normal temperature conditions. After 12 hours the stress was removed, and all cultures were grown at normal culturing conditions until Time = 48 hours when parasitaemia was measured again.

For the asynchronous oxygen stress assay, parasite cultures were distributed into 6 well plates and diluted with a target parasitaemia of 0.5%. Parasitaemia was measured for each well and this was designated Time = 0. Oxygen stress was applied to half of the cultures in the form of incubation with atmospheric air (21% O₂ by volume) for 6 hours to mimic oxidative stress. The other half cultures were grown at normal conditions. After 6 hours the stress was removed, and all cultures were grown under normal culturing conditions until Time = 48 hours when parasitaemia was measured again.

For the synchronised heat stress assay, parasites were highly synchronised by a combination of sorbitol treatment and enrichment with Percoll gradient. Specifically, parasites were initially synchronised using sorbitol to obtain a ring-stage culture then late-stage parasites in the same cycle were enriched using a Percoll gradient. Finally, 8 hours post Percoll selection, parasites were once again subjected to sorbitol treatment to obtain an ~8 hour window of synchronisation. The rest of the assay was performed as for the asynchronous assays but begun at different points in the blood stage life cycle.

The percentage change in parasitaemia (PCP) was calculated by:

$$PCP = \frac{P_{48} - P_0}{P_0} \times 100 \quad (2.5)$$

In which P_{48} is parasitaemia measured at 48 hours and P_0 at 0 hours.

2.7.4 Drug Assays

Compounds were dissolved in DMSO to create stocks at 10 mg mL⁻¹. Stock compounds were diluted in hypoxanthine-free culturing medium and titrated in 96-well plates in duplicate. Δ PfHsp70-x parasite infected erythrocytes were added to each well (except for negative control wells which contained uninfected erythrocytes) to a final parasitaemia of 0.3% and 1.25% hematocrit. Incubation time with drug pressure varied between experiments as indicated. For shorter drug pressure times, compounds were removed with thorough washing of cultures using hypoxanthine-free culturing medium. After 8 hours incubation, 0.25 μ Ci [³H]-hypoxanthine was added, and plates further incubated for 16 hours. Parasitized cells were lysed using freeze-thaw and then the lysed cultures were passed through glass-fibre filters trap to DNA. Radioactivity was counted using a MicroBeta2 plate liquid scintillation counter (PerkinElmer). The effect of compounds on parasite growth was calculated as a percentage of the untreated controls.

2.8 Graphs and Structures

All graphs and statistics in this thesis were plotted and calculated using R through R Studio. Structural models shown in this thesis were visualised with PyMOL [124].

3

Cell Biology

Contents

3.1	Contributions to Data	44
3.2	Introduction	45
3.3	PfHsp70-x knockout and knockdown cell lines	46
3.4	ΔPfHsp70-x susceptibility to antimalarials	48
3.5	Bromo-β Lapachol - the 'specific inhibitor of PfHsp70-x	53
3.6	The effect of stress on PfHsp70-x knockdown parasite	
	growth	55
3.6.1	Oxidative stress	55
3.6.2	Heat stress	56
3.6.3	Discrepancies between biological repeats	58
3.6.4	Heat stress through the parasite's life cycle	65
3.7	Discussion	68

3.1 Contributions to Data

Plasmodium falciparum 3D7 parasite lines WT, Δ PfHsp70-x, PfHsp70-x_{glmS} and PfHsp70-x_{M9} were a kind gift from Dr Vasant Muralidharan of the University of Georgia, USA [83]. All work in this chapter was completed at the Department of Medical Parasitology and Infection Biology at the Swiss Tropical and Public Health Institute, Basel thanks to the help of Prof. Hans-Peter Beck and his lab, especially Dr. Beatrice Schibler and Armin Passecker. The drug assays were made

possible by Prof. Matthias Rottman and carried out alongside Christin Gump. The majority of work presented here was completed by myself, except for the heat shock experiments using synchronised cells which were performed by Armin Passecker.

3.2 Introduction

Re-annotation of the *P. falciparum* genome resulted in the discovery of an exported Hsp70 previously thought to be a non-functional pseudogene [125]. PfHsp70-x, shown to localize to J-dots in the red blood cell (RBC) cytoplasm, was suggested to assist in folding, trafficking and protection of parasitic proteins within the host cell erythrocyte, thereby linking this chaperone to parasite survival and pathogenicity [71]. Recent work performed by Charnaud *et al* [82] tested a PfHsp70-x knockout (Δ PfHsp70-x) parasite line for a number of phenotypes associated with normal growth, response to stressful conditions, cell morphology and the export of virulent proteins such as PfEMP1. Their findings indicated that PfHsp70-x supports virulence functions as Δ PfHsp70-x parasites showed less efficient export of PfEMP1 with α -PfEMP1 antibodies binding reduced by 32% and reduced cytoadhesion (63% reduction compared to wildtype). Other effects were seen such as higher sensitivity to nutrient restriction and oxygen stress. This study highlighted however that knockout parasites had undergone a degree of selection that caused changes in the transcription and translation of multiple exported proteins such as RESA, a PHIST protein with a J domain. Such changes may be possibly compensating for the loss of PfHsp70-x and therefore mask phenotypes. Cobb *et al* [83] then published a study in which they had utilised CRISPR/Cas9 genome editing to create PfHsp70-x ‘knockdown’ parasite lines. They found the knockdown lines did not show a reduction in growth under normal conditions but did not test for response to stress.

In this chapter we utilize the conditional knockdown parasite lines of Cobb *et al* to study the effect of losing PfHsp70-x on a timescale too short for selection and thus possible compensation to occur. In particular, we focus on the role of PfHsp70-x in the parasite’s response to stress.

3.3 PfHsp70-x knockout and knockdown cell lines

The PfHsp70-x conditional knockdown mutant received from Dr. Muralidharan had been created by inserting a glmS ribozyme sequence into the 3' untranslated region (UTR) of the PfHsp70-x gene. The glmS ribozyme is transcribed alongside the PfHsp70-x gene as one mRNA and, upon addition of glucosamine (GlcN) to the culture medium, is able to cleave itself leading to loss of the poly(A) tail of mRNA. Poly(A) tail cleavage results in degradation of the PfHsp70-x mRNA, resulting in highly reduced protein expression and, thus, PfHsp70-x 'knockdown'.

To confirm the reduction of PfHsp70-x levels upon GlcN addition, Western blots were performed to compare knockdown and control cells after GlcN treatment. Asynchronous cultures of PfHsp70-x_{glmS} parasites and parasites where a non-functional version of the glmS ribozyme was inserted in the PfHsp70-x UTR (PfHsp70-x_{M9}), were split into two cultures with one of each treated with 2.5 mM GlcN for 48 hours to induce ribozyme self-cleavage and the other left in normal growth media. 48 hours was predicted to be sufficiently long as the parasite life cycle is ~48 hours and therefore all parasites present in culture at the 48-hour timepoint should have developed in the presence of GlcN. PfHsp70-x protein levels were probed with an α -PfHsp70-x antibody with the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) used as a loading control probed by an α -GAPDH antibody. As shown in Fig. 3.1, we can see that the PfHsp70-x_{M9} cell lysates contain similar levels of PfHsp70-x as to be expected of a negative control. In contrast, PfHsp70-x_{glmS} cell lysates show a significant reduction in PfHsp70-x levels upon GlcN addition, indicating the knockdown was successful.

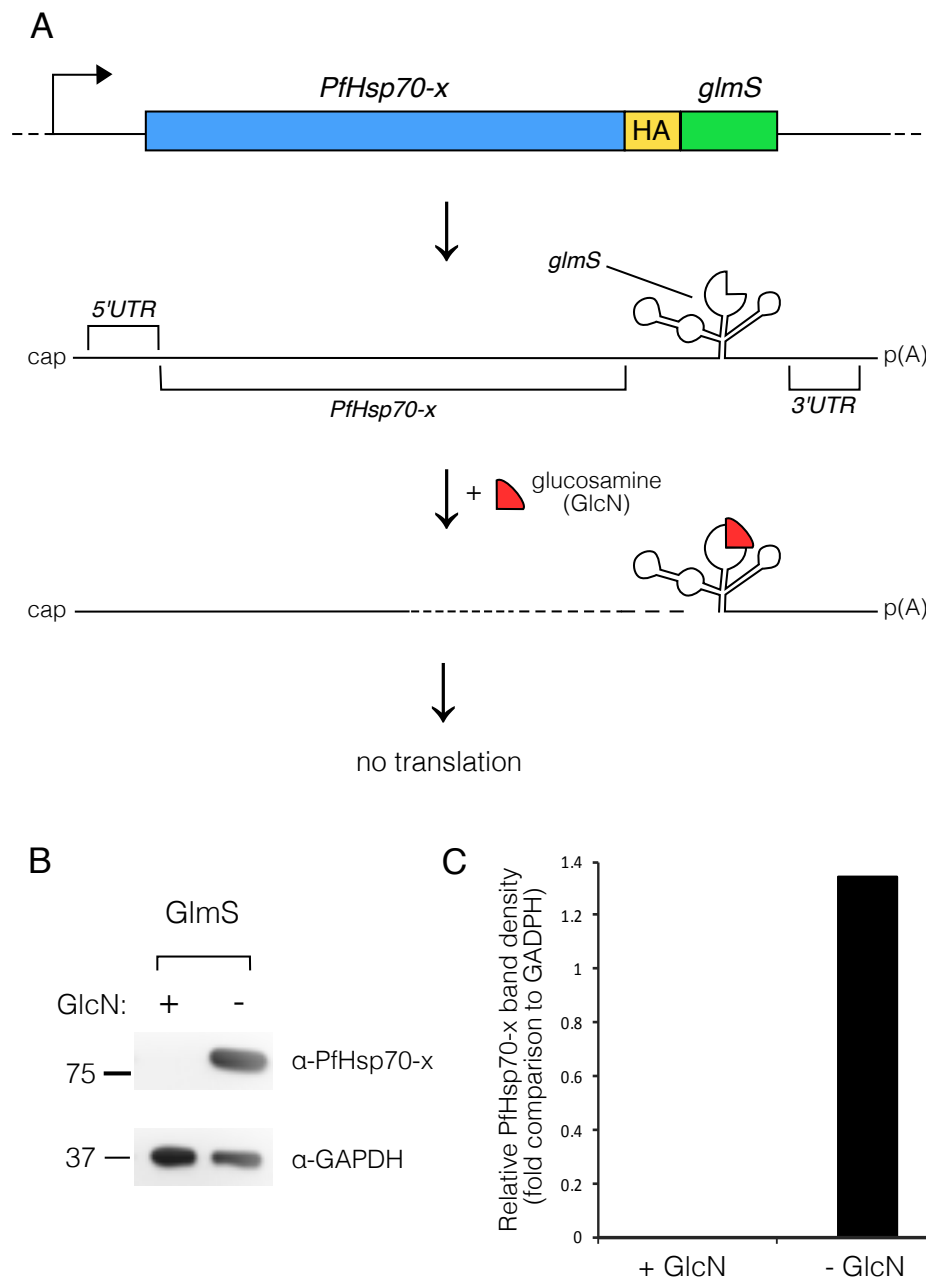


Figure 3.1: The GlmS conditional knockdown system. **A:** An illustration of the glmS conditional knockdown system. A HA tag and glmS sequence is inserted into the 3' untranslated region (UTR) of the gene of interest. The transcribed mRNA contains the GlmS ribozyme which, upon addition of glucosamine (GlcN), cleaves itself. GlmS ribozyme cleavage causes detachment of the poly(A) tail leading to mRNA degradation and knockdown of the protein of interest. **B:** Western blot to confirm successful knockdown in PfHsp70-x_{glmS} upon addition of glucosamine (GlcN) in comparison to the PfHsp70-x_{M9} control. Proteins were visualised using α-PfHsp70-x (1:5,000; [71]) and α-GAPDH antibodies (1:10,000; C. Daubenberger). **C:** Densitometry analysis of **B** to determine PfHsp70-x knockdown levels calculated using ImageJ. PfHsp70-x band density was compared to the loading control GAPDH to determine relative levels of protein with and without GlcN.

The PfHsp70-x knockout line (Δ PfHsp70-x) from Dr. Muralidharan was generated by interrupting the open reading frame by inserting a human dihydrofolate reductase (hdhfr) drug resistance cassette using CRISPR/Cas9. Parasites were selected with WR99210 and knockout parasites were cloned via limiting dilution. We used western blots to confirm that the PfHsp70-x knockout clones received did indeed did not express PfHsp70-x (Fig. 3.2). Two separate knockout clones were received ((Δ PfHsp70-x 1 and 2). After determining both did not express PfHsp70-x, we picked one line to continue working with.

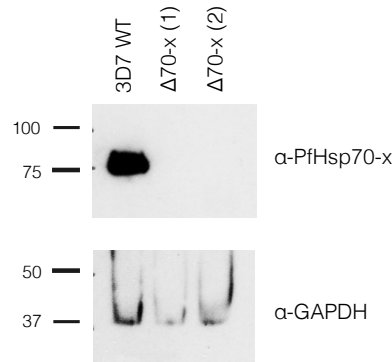


Figure 3.2: Δ PfHsp70-x confirmation. Western blot to confirm successful knockout of PfHsp70-x in comparison to the 3D7 wildtype (WT) parasites. Proteins were visualised using α -PfHsp70-x [71] and α -GAPDH (C. Daubenberger) antibodies.

3.4 Δ PfHsp70-x susceptibility to antimalarials

Previous studies did not explore the role PfHsp70-x played in protection from stress caused by antimalarials. A number of antimalarials such as artemisinin and the quinols are thought to act through the generation of free radicals although mechanisms have not been fully deduced. Some studies have shown that chaperones, including Hsp70s, play a role in protecting proteins from free radical damage and target irreversibly damaged proteins for degradation [126]. We wanted to test if PfHsp70-x played such a role in *P. falciparum* leading to reduced potency of antimalarials. If so, then a combined treatment therapy consisting of a traditionally used antimalarial alongside a PfHsp70-x inhibitor could be extremely efficient.

We performed *in vitro* experiments to compare antimalarial IC_{50} values between wildtype 3D7 and a PfHsp70-x knockout parasite line, Δ PfHsp70-x. Experiments were performed in 96-well plates to allow the study of several drugs in parallel (Fig. 3.3) and with asynchronous cultures. Δ PfHsp70-x and control (3D7) parasites were distributed across two plates with uninfected red blood cells (uRBCs) then drugs were added to cultures by serial dilution in duplicates. Drug pressure was applied for 1 or 8 hours depending on drug concentration and then removed through several washing steps. 3H hypoxanthine was then added and cultures were incubated for the remainder of a 24 hour period to allow hypoxanthine to be incorporated into the DNA. 3H hypoxanthine incorporation was used as a measure of growth and normalised against iRBCs that experienced no drug pressure and uRBCs. IC_{50} values were calculated by fitting a sigmoidal dose-response model to the data. A difference in IC_{50} was deemed significant if there was over a 2 fold change compared to the value for the wildtype parasite line due to the small number of technical repeats.

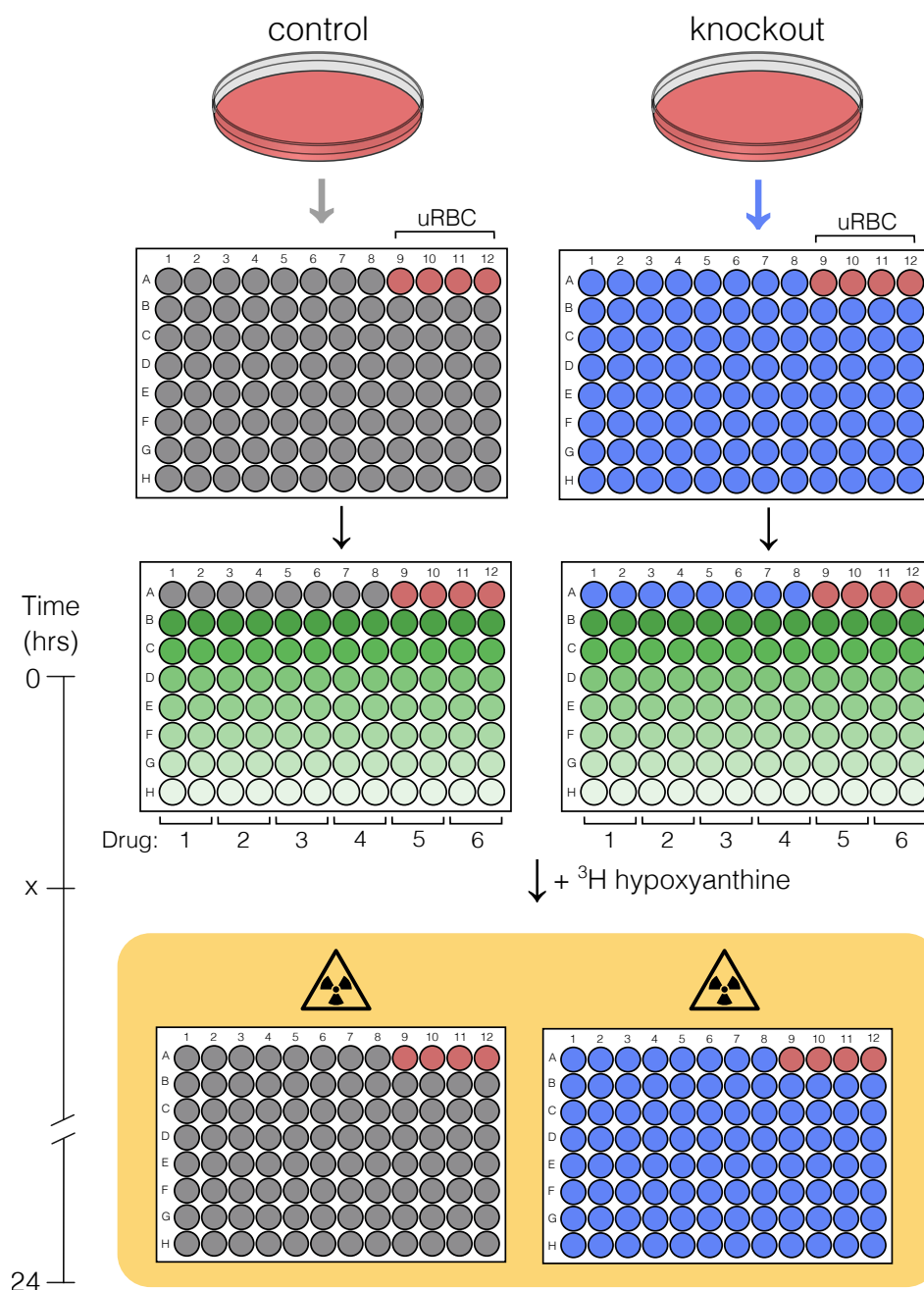


Figure 3.3: Antimalarial drug experiment schematic. Asynchronous cultures of control and knockout cell lines were plated separately on two 96-well plates with 4 wells left for the negative control of uninfected red blood cells (uRBC). Drugs (green) were added to each plate in duplicate columns and serially diluted to create a dose-response curve at time = 0 h and parasites were cultured in standard conditions. The top row was not treated with any drug to provide positive controls for growth. After time = x (8 or 1 h), drug pressure was removed through extensive washing and ^3H hypoxanthine was added to all wells. Hypoxanthine incorporation was allowed to progress for 24 - x h. Growth was then measured through hypoxanthine incorporation as a percentage of the positive controls corrected for the uRBC negative control.

The effect of antimalarials artesunate (an artemisinin derivative with improved solubility), lumefantrine, chloroquine and primaquine were studied because it has been reported that their antimalarial activity may be due to the production of free radicals. Although these antimalarials are believed to act primarily in the parasite, their free radical activity may begin in the host cell cytoplasm or alternatively, free radicals generated in the parasite may lead to oxidative stress in the host cell cytoplasm. Artesunate is the currently the first line treatment for patients with severe malaria [127]. Chloroquine is also administered extensively for the treatment of malaria caused by all *Plasmodium* species as well as its prevention although resistance to the drug has spread to nearly all areas of the world where *falciparum* malaria is transmitted [128]. Lumefantrine is commonly used as a co-drug in combination with artemisinin [129, 130] Primaquine is often used in the prevention of *P. vivax* infection relapse but has also been found to be effective against certain stages of *P. falciparum* [11].

Initially, assays were performed with 8 hours of drug pressure applied (Fig. 3.4). We can see that both artesunate and chloroquine are potent drugs with IC_{50} values of $\sim 8 \mu M$ and $95 \mu M$ respectively comparable to values measured previously in the lab of Prof. Rottman. Primaquine is less potent with IC_{50} value of $\sim 7700 \mu M$ and an IC_{50} value for lumefantrine could not be calculated as drug concentrations were not high enough. We can see that for all drugs tested, no significant difference could be seen between IC_{50} values for WT and $\Delta PfHsp70$ -x parasites. It was postulated that the timescale of 8 hours of drug pressure was too long to see any possible differences between $\Delta PfHsp70$ -x and WT parasites as artesunate and chloroquine in particular are fast acting drugs. The experiment was therefore repeated with just 1 hour of drug pressure and higher drug concentrations (Fig. 3.5). Primaquine was not repeated as higher concentrations could not be achieved due to solubility issues.

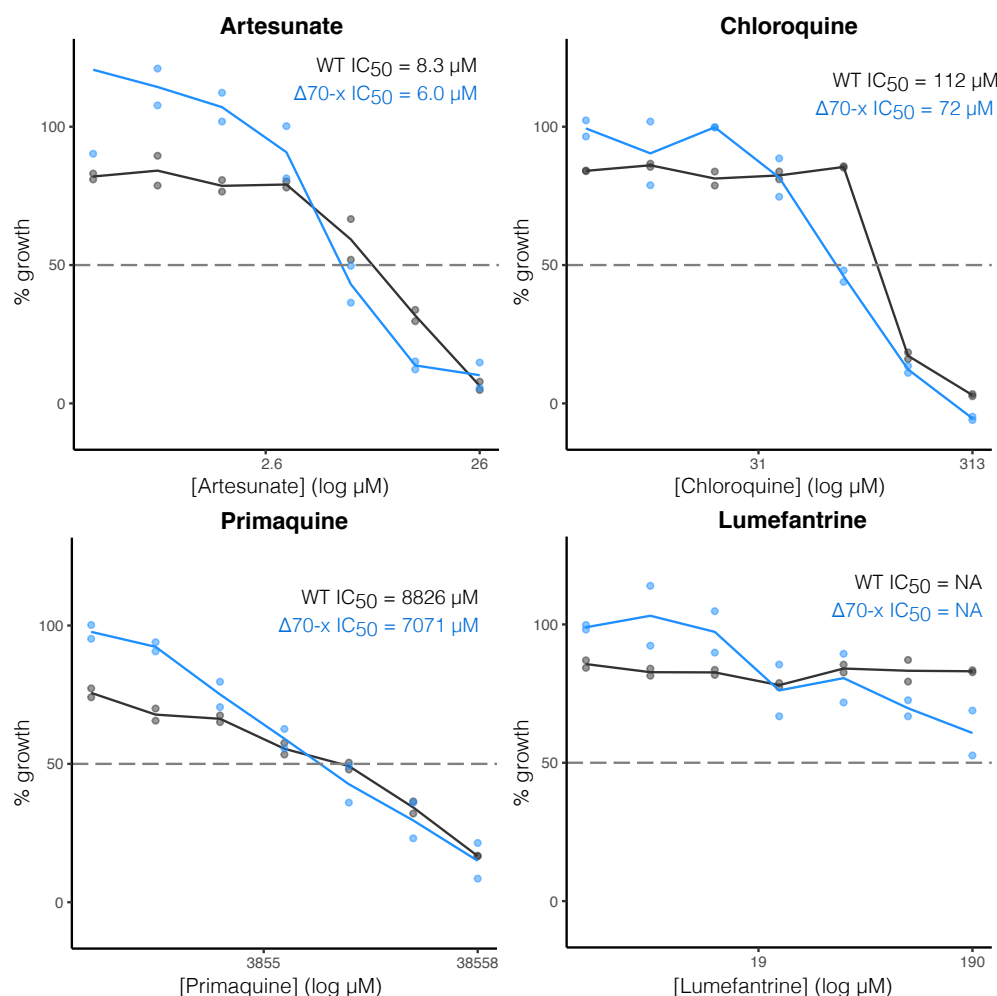


Figure 3.4: Dose-response curves for 8 h antimalarial treatment. Plots of % growth of iRBCs compared to untreated iRBCs against drug concentration. Each drug concentration was tested in duplicate with both points shown. Line runs through the mean of the duplicates. IC_{50} values for each cell line wildtype (WT) and Δ PfHsp70-x ($\Delta 70-x$) are indicated on each plot.

The 1-hour drug pressure experiment resulted in higher IC_{50} values, particularly for artesunate, which may cause previously unobserved differences to be magnified and thus be observable. With the higher concentrations of lumefantrine, we were able to calculate IC_{50} values of $\sim 570 \mu$ M. For both artesunate and lumefantrine, almost identical IC_{50} values are calculated for both WT and Δ PfHsp70-x parasites. Interestingly, we see a difference in IC_{50} for chloroquine in which the Δ PfHsp70-x appears to be more sensitive to the drug with an IC_{50} of 220μ M compared to 370μ M for the WT.

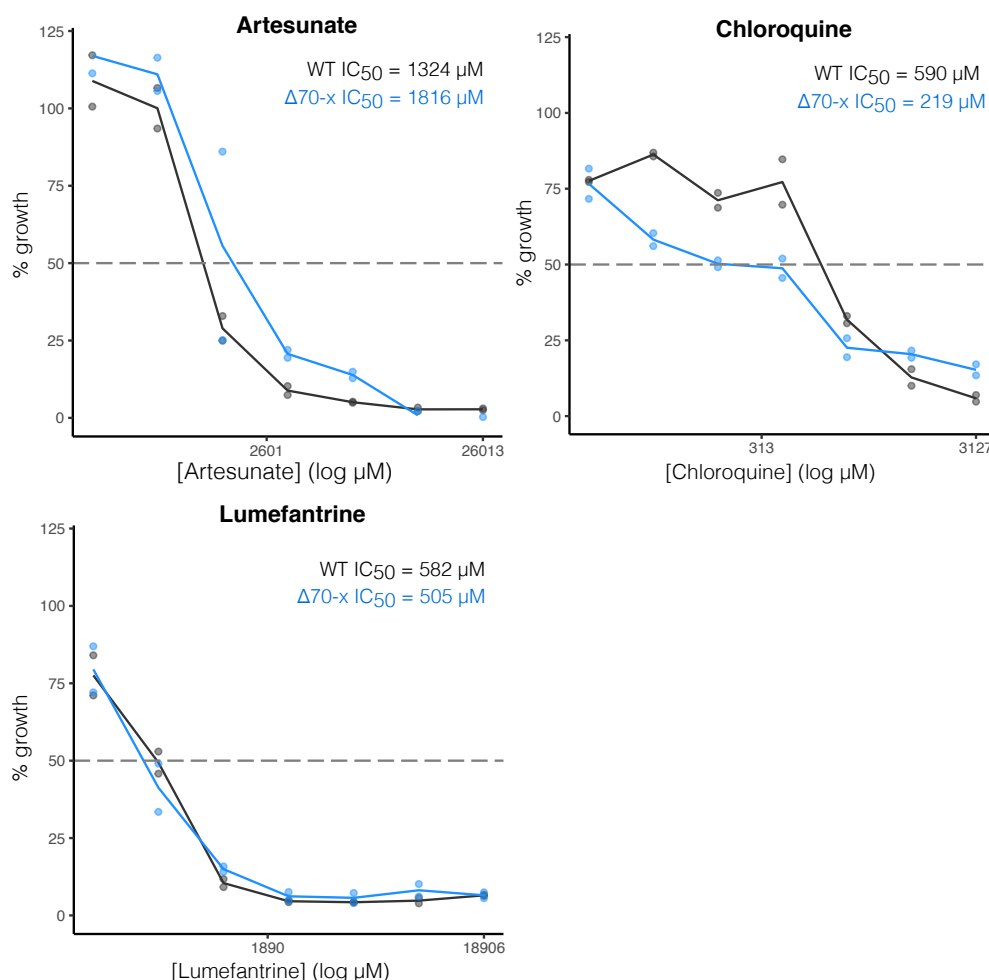


Figure 3.5: Dose-response curves for 1 h antimalarial treatment. Plots of % growth of untreated iRBCs against drug concentration. Each drug concentration was tested in duplicate with both points shown. Line runs through the mean of the duplicates. IC_{50} values for each cell line wildtype (WT) and $\Delta PfHsp70-x$ ($\Delta 70-x$) are indicated on each plot.

3.5 Bromo- β Lapachol - the 'specific inhibitor of PfHsp70-x

Cockburn *et al* reported the discovery of the compound, bromo- β lapachol (BBL), which has antimalarial activity (IC_{50} of $17 \mu M$) and found BBL able to preferentially inhibit the activity of PfHsp70-x when compared to the intraparasitic Hsp70 (PfHsp70-1) and human Hsp70 (Hsp72) [131, 132].

Alongside the antimalarial drug assays, we also studied the effect of BBL on $\Delta PfHsp70-x$ parasites in comparison with WT parasites to test for the aforemen-

tioned specificity (Fig. 3.6). We see similar IC_{50} values for both Δ PfHsp70-x and WT parasite lines despite the reported specificity. This suggests that BBL has off-target effects and if anything, PfHsp70-x protects the parasite. The mechanism by which BBL inhibits PfHsp70-x remains unclear and needs further investigation to determine the nature of this inhibition and to further understand its antimalarial activity.

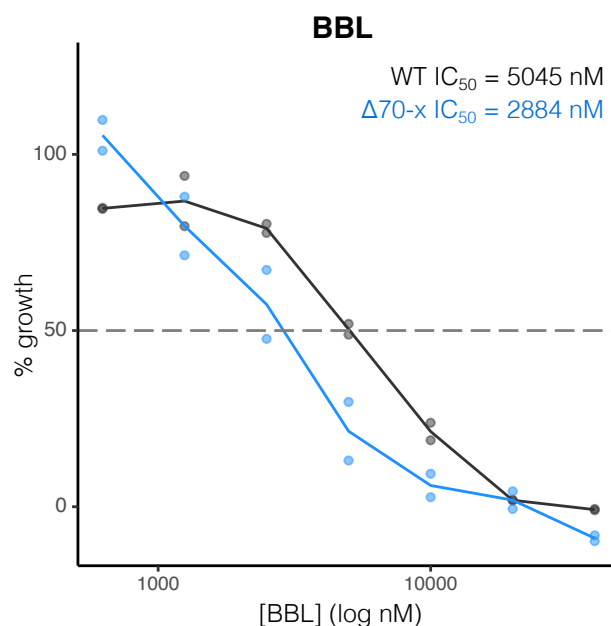


Figure 3.6: Dose-response curves for BBL treatment. Plots of % growth of untreated iRBCs against drug concentration. Each concentration was tested in duplicate with both points shown. Line runs through the mean of the duplicates. IC_{50} values for each cell line wildtype (WT) and Δ PfHsp70-x ($\Delta 70-x$) are indicated on the plot.

However, it is important to note that these assays were performed with a Δ PfHsp70-x line that may have experienced changes in gene expression during the knockout generation similar to that found in the Charnaud study. Such changes in gene expression may cause phenotype masking and thus prevent us determining the role of PfHsp70-x in response to antimalarials. To overcome possible changes in gene expression, the following assays utilise the conditional PfHsp70-x knockdown line, PfHsp70-x_{glmS} which involves rapid yet significant reduction in protein which prevents selection of compensated parasites.

3.6 The effect of stress on PfHsp70-x knockdown parasite growth

A number of experiments were executed to test the growth of the rapid PfHsp70-x knockdown cell lines after exposure to stresses. We hypothesise that the PfHsp70-x knockdown line will show greater sensitivity to stress than control parasites indicating that PfHsp70-x plays a role in protecting the parasite. Here we focus on the stressors oxygen and heat.

3.6.1 Oxidative stress

An experiment was designed in which we compare the growth of asynchronous cultures of control PfHsp70-x_{M9} with knockdown PfHsp70-x_{glmS} after a period of oxidative stress. We mimic oxidative stress by culturing in atmospheric air which has an oxygen content of ~21%. *P. falciparum* parasites, and indeed other *Plasmodium* species, prefer low oxygen environments for growth which is why parasites are cultured in a special gas mixture normally consisting of 3% O₂, 5% CO₂ and 92% N₂.

GlcN was applied to parasite cultures 48 hours prior to experiment setup to ensure sufficient knockdown of PfHsp70-x. Parasitaemia was measured at the assay start and after 48 hours using flow cytometry with the oxygen stress applied between 0 – 6 hours (Fig. 3.7). All cultures were grown in normal conditions for the remaining 42 hours. The experiment was performed with three technical repeats and three biological repeats. Parasite growth was calculated as percentage change from the initial parasitaemia and averaged across technical replicates. Growth for stressed parasites was then normalised using the growth of unstressed parasites.

An unpaired T-test was used to compare the mean growth across biological repeats between PfHsp70-x_{glmS} knockdown and PfHsp70-x_{M9} control cell lines after a period of oxygen shock. With a p-value of 0.2974 we found no statistically significant difference in growth, and therefore no effect of lacking PfHsp70-x in response to oxidative stress (Fig. 3.7).

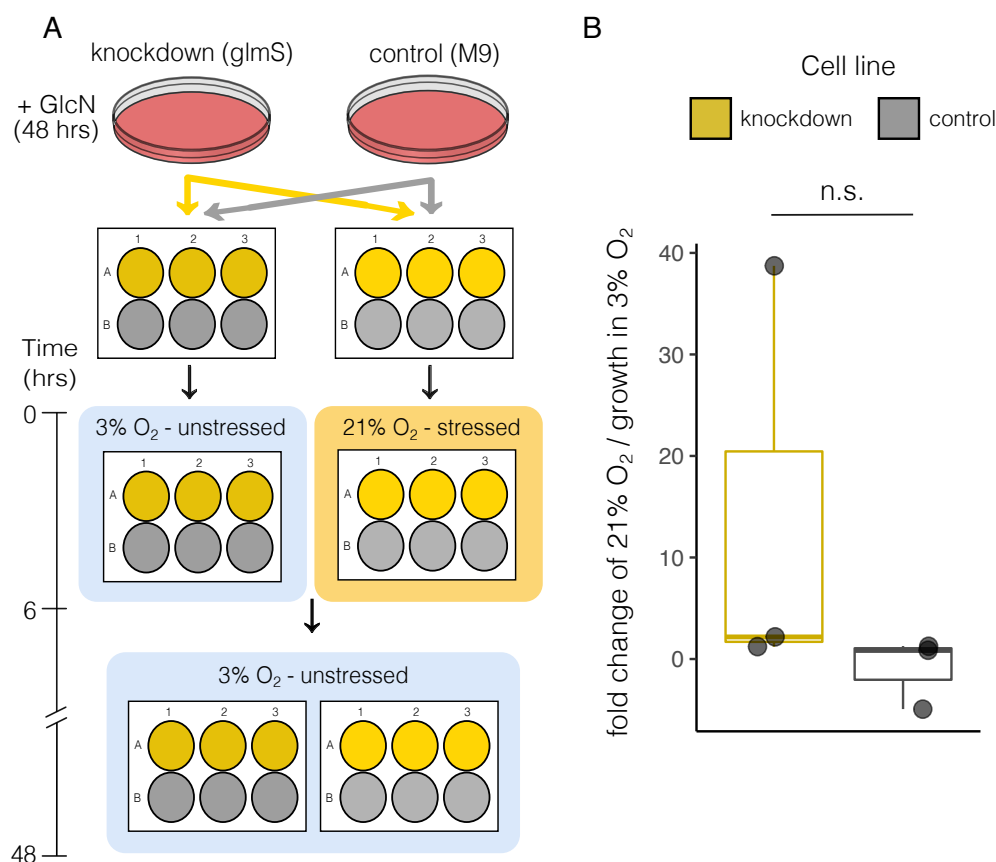


Figure 3.7: Oxidative stress growth experiment. **A:** Asynchronous control and knockdown cell lines were split between two 6-well plates and parasitaemia measured at 0 hours. One plate was incubated in atmospheric air consisting of 21% O₂ for 6 hours. After 6 hours both plates were cultured in standard conditions for the next 42 hours. Final parasitaemia was measured at 48 hours. **B:** Boxplot comparing growth of PfHsp70-x_{glmS} knockdown and PfHsp70-x_{M9} control lines after oxidative stress. Growth is expressed as a percentage of growth in standard conditions. Each point represents the mean of 3 replicates for a biological repeat. The difference in mean growth was calculated as not significant (n.s.) using an independent T-test (p-value = 0.297).

3.6.2 Heat stress

A similar experimental setup was used to explore the effect of the PfHsp70-x knockdown in response to heat stress (Fig. 3.8). The parasite experiences heat stress during periods of fever during the intraerythrocytic stage of infection. Asynchronous cultures of PfHsp70-x_{M9} and PfHsp70-x_{glmS} were split with half subjected to a heat shock of 40 °C for 12 hours to mimic fever and the other half cultured in normal conditions. All cultures were grown in standard conditions for the remaining 36 hours. The experiment was performed with three technical replicates and three biological

repeats, and growth calculated as discussed above for the oxidative stress experiment.

An unpaired T-test was used to determine if parasite growth was significantly different between PfHsp70-x_{glmS} knockdown and PfHsp70-x_{M9} control cell lines after a period of heat shock (Fig. 3.8). With a p-value of 0.9103 we observed no significant differences, and therefore observed no effect of lacking PfHsp70-x in response to heat shock.

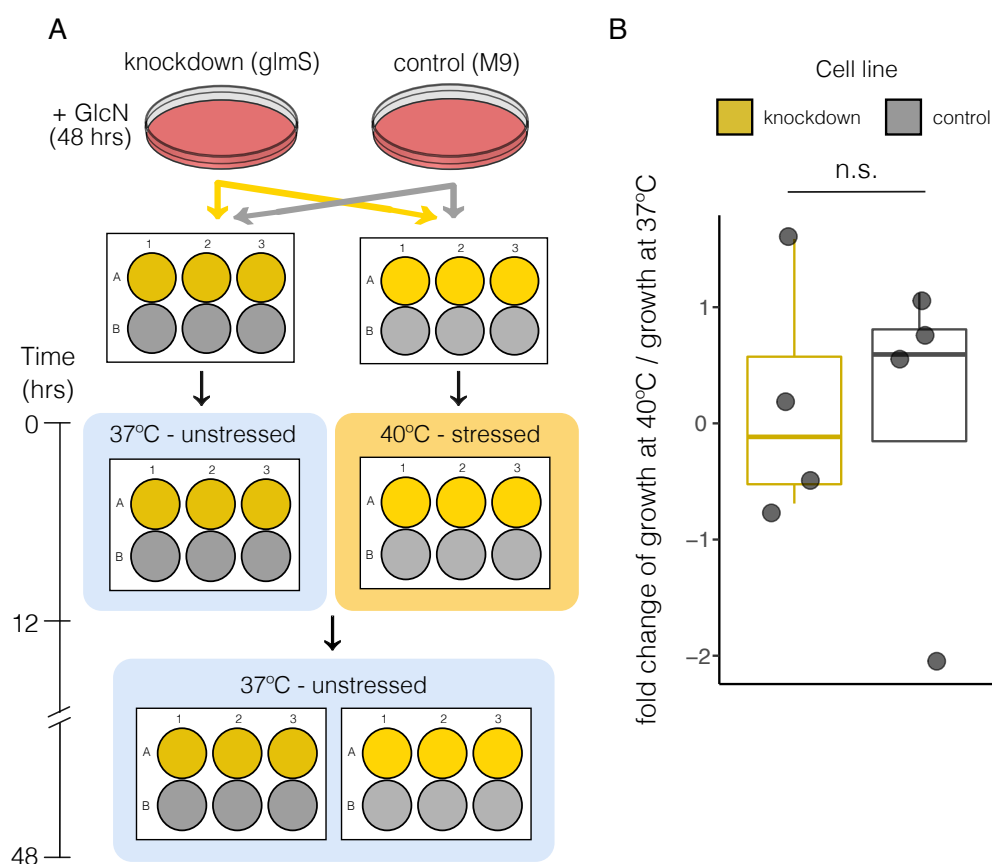


Figure 3.8: Heat stress growth experiment schematic. A: Asynchronous control and knockdown cell lines were split between two 6-well plates and parasitaemia measured at 0 hours. One plate was incubated at 40 °C for 12 hours. After 12 hours both plates were cultured in standard conditions for the next 36 hours. Final parasitaemia was measured at 48 hours. **B:** Boxplot comparing growth of PfHsp70-x_{glmS} knockdown and PfHsp70-x_{M9} control lines after heat stress. Growth is expressed as a percentage of growth in standard conditions. Each point represents the mean of 3 replicates for a biological repeat. The difference in mean growth was calculated as not significant (n.s.) using an independent T-test (p-value = 0.910).

3.6.3 Discrepancies between biological repeats

Parasites lacking PfHsp70-x showed no significant difference in viability and growth in response to heat and oxygen stress compared to a control cell line. We do however see large variability in our data for both cell lines in both heat and oxygen stress experiments. By looking at each biological repeat we can see that repeats have vastly different growth behaviours. For example, we see parasite growth rates are far higher in biological repeat 3 for the heat shock experiment than in other biological repeats (Fig. 3.10). The same can be said for biological repeat 1 knockdown cells in comparison to other repeats in the oxygen stress experiment (Fig. 3.9). It is the variability across biological repeats, despite similar initial parasitaemia values, that make it difficult to pull out meaningful and reproducible differences.

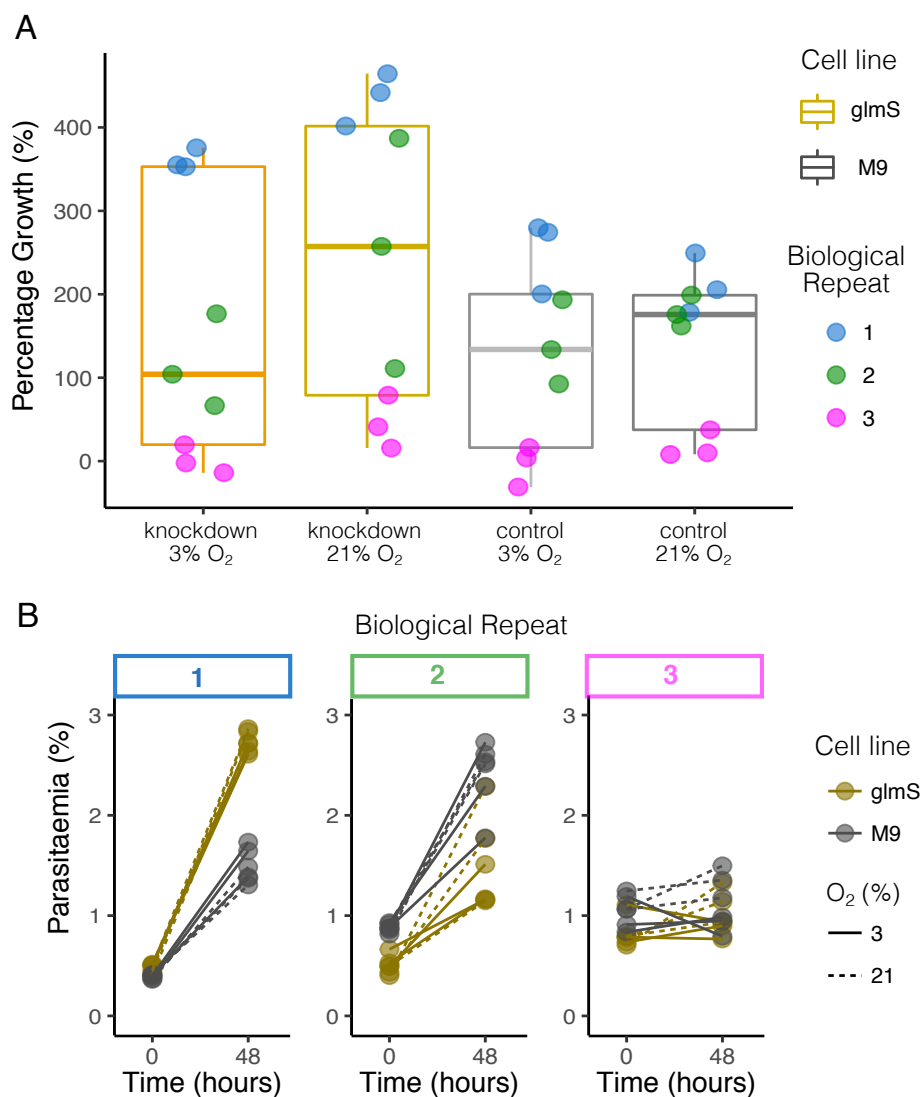


Figure 3.9: Oxidative stress growth experiment variability. **A:** Boxplot comparing growth of PfHsp70-x_{glmS} knockdown and PfHsp70-x_{M9} control lines with and without oxidative stress. Each point represents a technical replicate. Growth is shown as a percentage of initial parasitaemia. **B:** Boxplot comparing growth of PfHsp70-x_{glmS} knockdown and PfHsp70-x_{M9} control lines with and without oxidative stress with points coloured by biological repeat. **C:** A breakdown of each biological repeat showing the growth of each technical repeat.

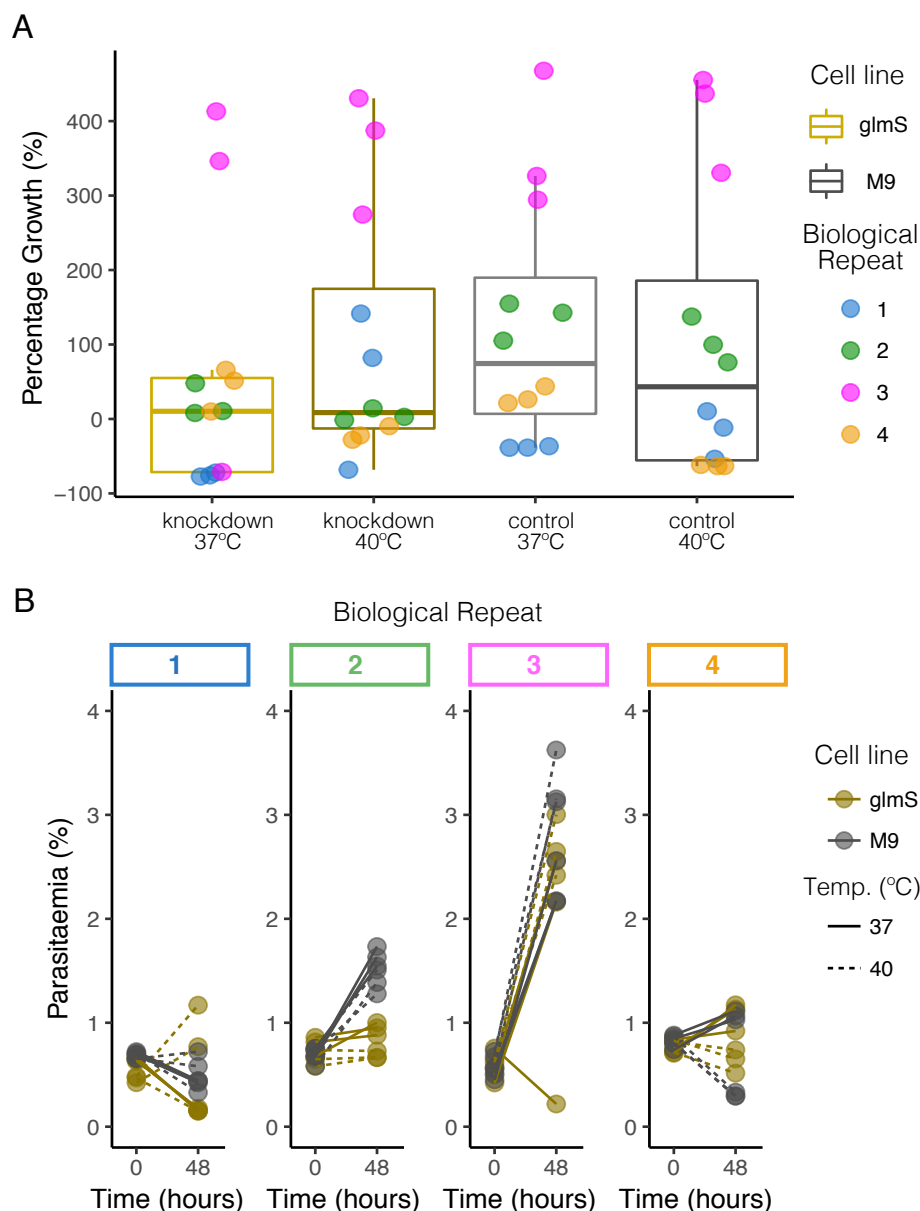


Figure 3.10: Heat stress growth experiment variability. **A:** Boxplot comparing growth of PfHsp70-x_{glmS} knockdown and PfHsp70-x_{M9} control lines with and without heat stress. Each point represents a technical repeat. Growth is shown as a percentage of initial parasitaemia. **B:** Boxplot comparing growth of PfHsp70-x_{glmS} knockdown and PfHsp70-x_{M9} control lines with and without heat stress with points coloured by biological repeat. **C:** A breakdown of each biological repeat showing the growth of each technical repeat.

To understand the discrepancies seen between each biological repeat, we delved deeper into the raw data collected. At each time point, parasitaemia was measured using flow cytometry and SYBR staining of DNA (Fig. 3.11). Uninfected red blood

cells (uRBCs) have no nucleus and therefore are not stained unlike infected red blood cells (iRBCs) which contain the DNA of the parasite. Each flow cytometry reading consists of scattering and fluorescence information for each event i.e. each cell. Scattering allows the characterisation of size and granularity which is used to gate intact cells. Cells are then gated on the basis of fluorescence which relates to the DNA content giving the percentage of iRBCs in the total population of cells. We can then look closer at the iRBC population and see that we have a distribution of fluorescence rather than the tight cluster we see for uRBCs. The distribution of fluorescence is due to the asynchronous nature of cultures used – the population consists of early- and late-stage parasites with few and many chromosomes respectively. As the parasite enters the mid- to late-stage of the intraerythrocytic cycle, DNA is copied, and new chromosomes are created (Fig. 3.12). A single parasite can create up to 32 merozoites with a large degree of variability which causes complications to the reading of flow cytometry data unlike for mammalian cells which have well-defined stages and a simple doubling of DNA content.

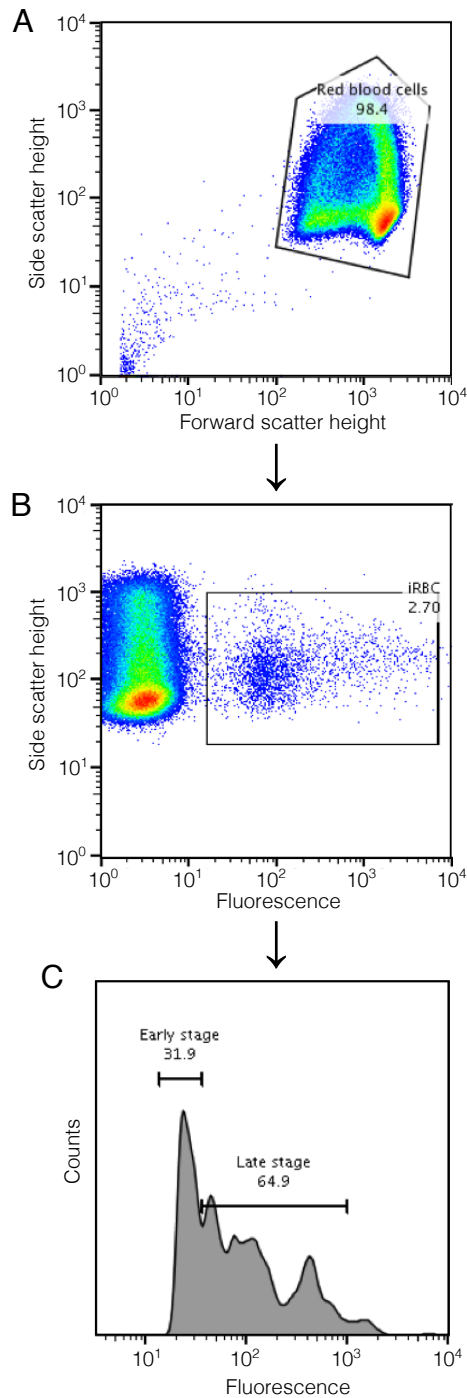


Figure 3.11: Flow cytometry of *P. falciparum* parasites **A:** Gating of red blood cells by light scattering. **B:** Further gating using fluorescence intensity from SYBR green staining of DNA to determine parasitaemia. **C:** Histogram of gated iRBCs showing distribution of fluorescence intensity and therefore DNA content in the population.

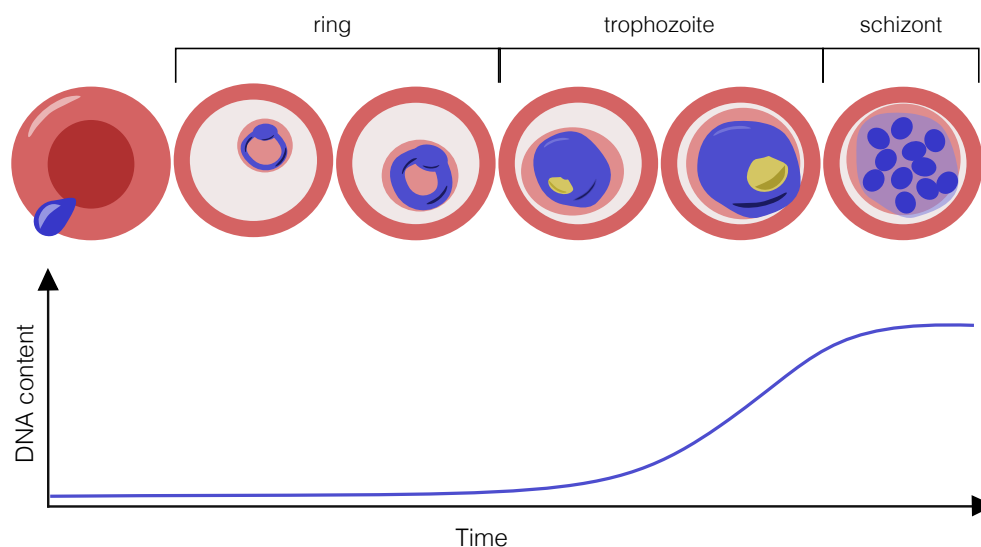


Figure 3.12: DNA content of *P. falciparum* throughout the intraerythrocytic life cycle. The intraerythrocytic life cycle of the *P. falciparum* parasite (top) and the associated increase in DNA content through the late trophozoite stage and schizogony (bottom) [133].

Previous attempts to accurately define the population of asynchronous cultures of *Plasmodium* parasites by flow cytometry using DNA content alone have so far been mostly unsuccessful and outside the scope of this thesis. However, we can qualitatively look at the distribution of fluorescence to be able to compare the distribution of parasite stages across biological repeats. One possible hypothesis for growth discrepancies under stress is that stress may affect the blood stage life cycle timing; for example, if the ring stage is prolonged because of the stress then this group will be overrepresented in the stressed samples compared to the unstressed. An alternative hypothesis is that loss of PfHsp70-x causes more significant growth differences in certain life cycle stages, and therefore that the growth effect varies depending on the make-up of the asynchronous culture.

By comparing the fluorescence distributions at time 0 h and 48 h for both unstressed and stressed conditions (Fig. 3.13), we can look for evidence that heat shock caused a change in intraerythrocytic life cycle progression. For both PfHsp70-x_{glnS} and PfHsp70-x_{M9}, we see that the fluorescence distribution at 48 hours is very similar whether there has been heat stress applied or not; thus, confirming that stress does not affect the cycle timing.

We do however see that the population make-up differs between different biological replicates (Fig. 3.14). In biological repeat 3, PfHsp70-x_{gImS} has a greater number of late stage parasites (higher fluorescence) than in biological repeat 2. It is also apparent that sometimes there are similar populations between the two cell lines, such as in biological repeat 2, but very different distributions in other biological repeats. Thus, it is likely that differences in initial populations are indeed causing the variability we see between biological repeats, and that the effect of stress in different cell stages are averaged out in the mixed population.

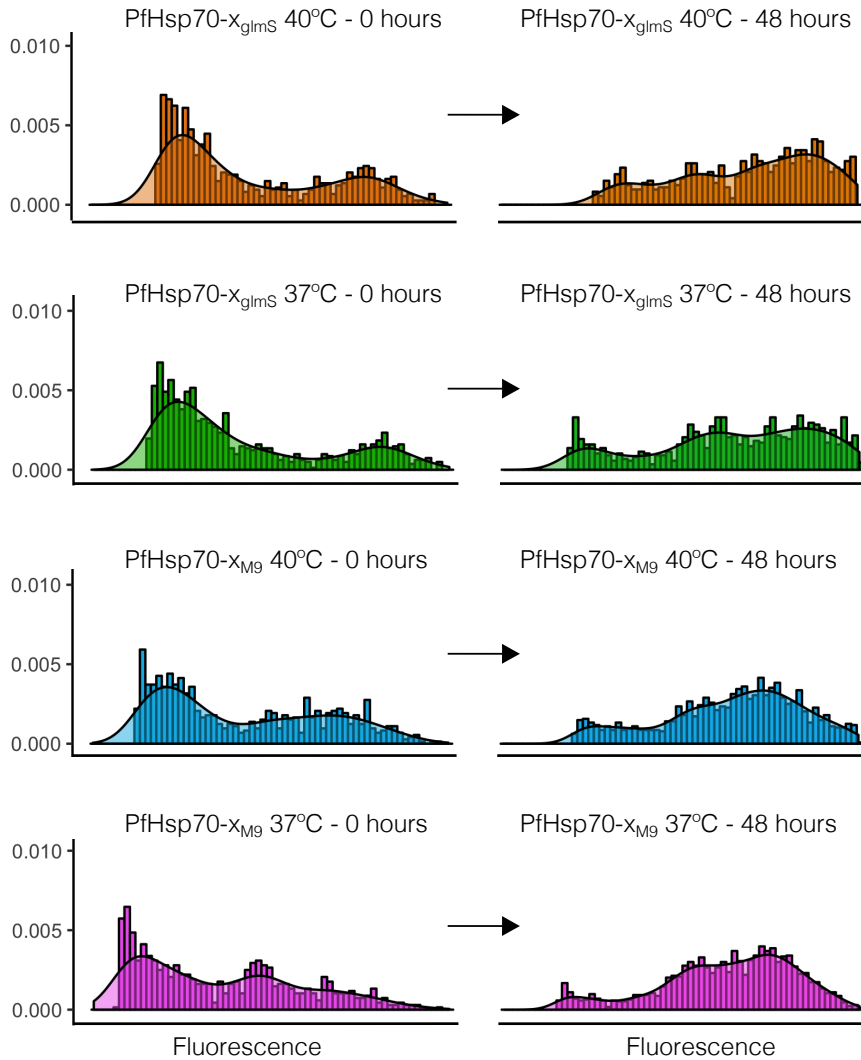


Figure 3.13: The effect of heat stress on life cycle progression. A comparison of fluorescence distribution of PfHsp70-x_{gImS} and PfHsp70-x_{M9} iRBCs at time = 0 h and 48 h with and without heat stress. Data shown is representative of technical replicates in each case.

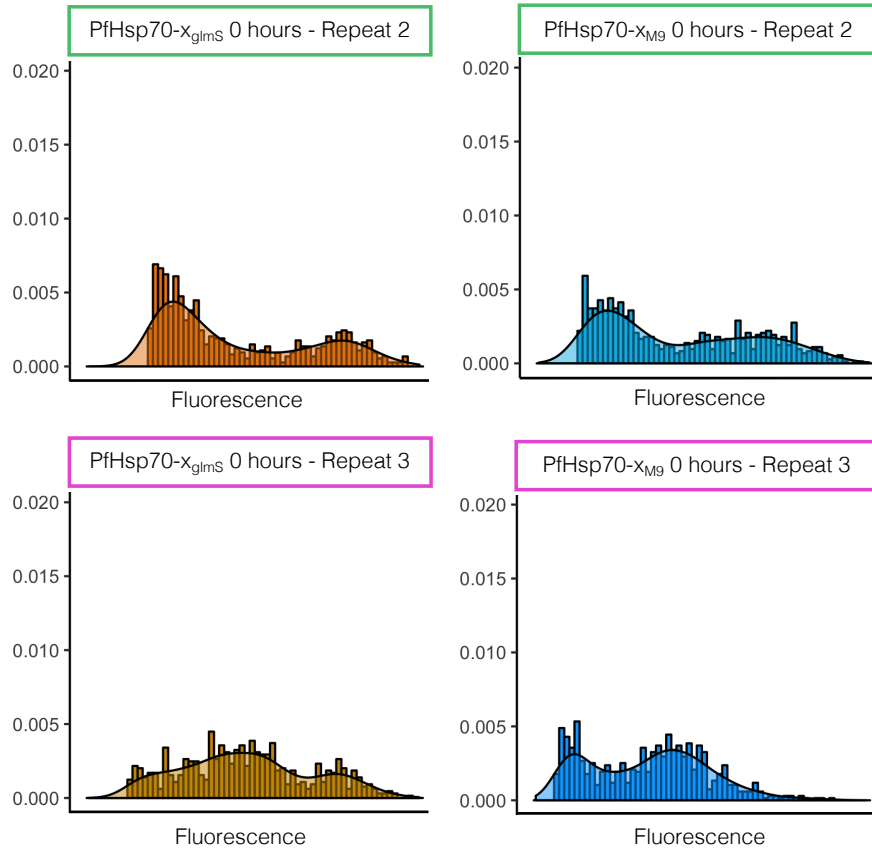


Figure 3.14: Initial population make-up of asynchronous cultures. A comparison of initial fluorescence distribution at time = 0 h between biological repeats 2 and 3 for both PfHsp70-x_{glmS} and PfHsp70-x_{M9}. Data shown is representative of technical replicates in each case.

3.6.4 Heat stress through the parasite's life cycle

To test the hypothesis that different stages of the parasite's cell cycle will be affected differentially by heat shock and therefore that PfHsp70-x functions are more important at different stages, we repeated the heat experiment with synchronised parasites and applied the 12 hour heat shock at various points throughout the cell cycle: young rings (0-12 hours), late rings (12-24 hours), trophozoites (24-36 hours) and late trophozoites and schizonts (36-48 hours). Only the PfHsp70-x_{glmS} parasite line was used to prevent comparing across different cultures and thus different populations. The control for this experiment was PfHsp70-x_{glmS} without GlcN. Comparing between GlcN present and absent conditions is a limitation of this experiment as it is difficult to rule out the possible effect of GlcN on results.

However, this limitation was deemed a necessary compromise to allow for comparison between identical populations as variation in population distribution caused issues in previous iterations. This experiment was performed with three technical replicates and three biological repeats. Growth of stressed parasites was normalised against growth of the same culture at standard conditions i.e. 37 °C. Independent T-tests were used to test for significance when comparing growth means of PfHsp70-x knockdown and control lines after heat shock.

We can see that the effect of the heat shock varies depending on which point in the life cycle the heat shock occurs (Fig. 3.15). Looking at the growth of the control culture alone, we can see that heat shock is beneficial to growth (mean = 1.7 fold increase of unstressed growth) when applied to young rings, but is detrimental during the trophozoite stage and has no significant effect when applied to late rings or late trophozoites/schizonts.

Interestingly, when comparing growth of control and PfHsp70-x knockdown parasites, we see a significant difference particularly when heat shock is applied in the young ring stage; we see increased growth for the control but not for the PfHsp70-x knockdown. This suggests that PfHsp70-x is crucial for accelerated growth in early ring stages during heat shock. It is important to note that heat shock during this stage is the most biologically significant as fever begins upon schizonts rupturing and lasts for several hours through reinfection and the ring stage [134]. It has also been previously reported that these periods of fever help the ring stages to proliferate and are important for the highly synchronised nature of the erythrocytic stage of infection [79].

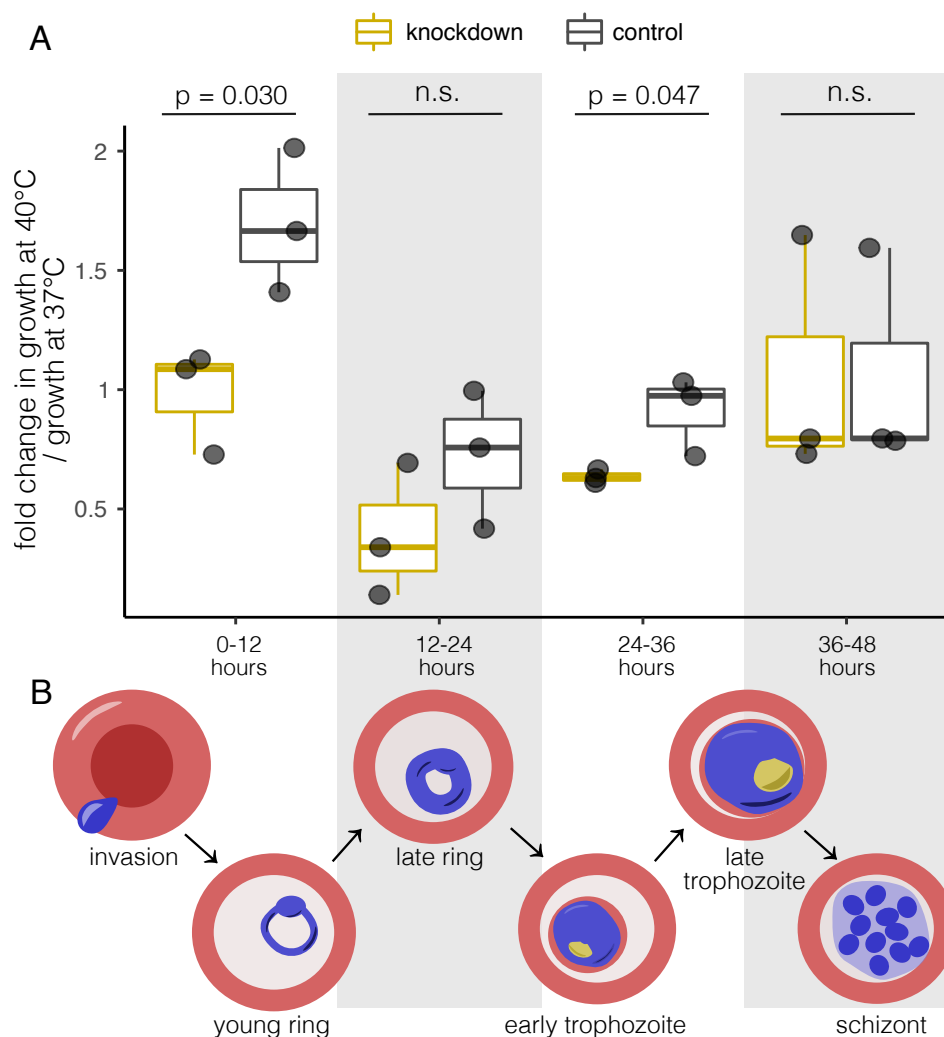


Figure 3.15: Heat stress growth experiment with synchronised parasites A: Boxplot comparing growth of PfHsp70-x_{glmS} with glucosamine (knockdown) and without glucosamine (control) after heat stress at different points in the life cycle. Growth is expressed as a percentage of growth in standard conditions i.e. 37°C. Each point represents the mean of 3 technical replicates. Significance between control and knockdown growth means was calculated by an independent unpaired T-test and the results indicated for each time window by either no significance (n.s.) or p-value if < 0.05 and thus significant. **B:** A schematic to demonstrate intraerythrocytic life stages being heat shocked.

A significant difference (p-value = 0.047) was also seen when heat shock was applied in the trophozoite stage. Growth of control parasites remains almost unchanged (mean = 91% of growth at 37°C) but PfHsp70-x knockdown parasites showed reduced growth (64% of growth at 37°C) suggesting that PfHsp70-x also plays a role in protecting the parasite from stress. A similar trend was seen for the 12-24 hour time window but the difference in growth of the control and PfHsp70-x

knockdown lines was not deemed significant. The lack of significance for this cell cycle stage may be due to variability seen between biological repeats and requires additional repeats to confirm if there is a relationship between the presence of PfHsp70-x and response to heat shock.

3.7 Discussion

In this chapter, we aimed to explore the role of PfHsp70-x in parasite viability in response to heat, oxidative and drug-induced stress.

We tested the response of a PfHsp70-x knockout (Δ PfHsp70-x) line to antimalarials thought to create free radicals and thus cause oxidative damage to the parasite. It was found that Δ PfHsp70-x was not more sensitive to artesunate or lumefantrine than the wildtype line. At high concentrations and short drug pressure, chloroquine did have a lower IC_{50} value for Δ PfHsp70-x than for the control suggesting that PfHsp70-x, to some degree, protects against chloroquine activity. Chloroquine is thought to act by preventing heme biocrystallisation resulting in the build-up of toxic, soluble heme within the parasite [128]. A combination of chloroquine and a PfHsp70-x inhibitor may make an interesting treatment option. However, it is possible that the knockout line used in the drug-stress assays experienced changes in expression during parasite line generation as was shown to be the case for the line used by Charnaud *et al.*

PfHsp70-x also makes an attractive drug target because Hsp70 chaperones have been previously targeted in the context of treating cancer and neurodegenerative diseases [135, 136, 137, 138, 139]. It therefore may be possible to repurpose existing Hsp70 inhibitors as antimalarials or use compounds as scaffolds for creating specific inhibitors.

Previous efforts to create a specific inhibitor of PfHsp70-x with antimalarial activity resulted in the discovery of several compounds by Cockburn *et al.* Here we test the activity of BBL, a supposedly specific inhibitor of PfHsp70-x, on a PfHsp70-x knockout line in comparison with wildtype 3D7 parasites. It was found that BBL retained its antimalarial activity in the absence of its proposed target. The

mechanism through which BBL inhibits PfHsp70-x's chaperone activity is unknown so further work needs to be carried out to understand how this compound functions.

Initial experiments testing the effects of heat and oxidative stress on a PfHsp70-x conditional knockdown line gave great variability between biological repeats preventing conclusions from being drawn. Upon delving deeper into the raw flow cytometry data, it was decided that this was due to the different initial distribution of life cycle stages in each population as experiments were performed with asynchronous cultures.

To determine the role of PfHsp70-x in response to heat stress, we repeated the heat stress experiments with synchronised parasites and applied stress at different points in the intrerythrocytic life cycle. We have shown for the first time that the exported chaperone PfHsp70-x plays an important role in the proliferation of *P. falciparum* under stress during the young ring stage (0-12 hours) and in the survival of the parasite in later-stage parasites (24-36 hours).

Of particular biological interest, is the role of PfHsp70-x in the proliferation of ring-stage parasites during heat shock. We saw that control parasites showed a significant increase in growth upon heat stress in the young ring stage but that this additional growth was not seen in the knockdown line. It is well known that acute fever in the malaria infected individual repeats in ~48 hour cycles coinciding well with the parasites life cycle [134, 140]. The rupture of schizonts, each releasing tens of merozoites into the bloodstream, has been shown to trigger fever that continues for 2 - 6 hours reaching temperatures of 41 °C. Previous work has shown that young ring-stage parasites may benefit from these febrile episodes by accelerated growth, increased cytoadherence and reduced deformability of the iRBC leading to increased sequestration in microvasculature [141, 79, 142, 80, 143]. Indeed, we see here that control parasites do grow faster after heat shock is applied to the ring stages. It is also interesting to note that PfHsp70-x transcription peaks at ~8 hours post-invasion correlating with the timing of fever (Fig. 3.16).

As previously discussed, comparing PfHsp70-x_{glnS} on and off GlcN has its limitations as it is difficult to rule out GlcN being the cause of any effect seen.

This was deemed to be a necessary compromise which allows for more accurate comparisons in terms of life cycle distribution in populations. The lack of difference in certain life cycle stages suggests that it is not GlcN itself that is causing differences in growth but a real result of the PfHsp70-x knockdown.

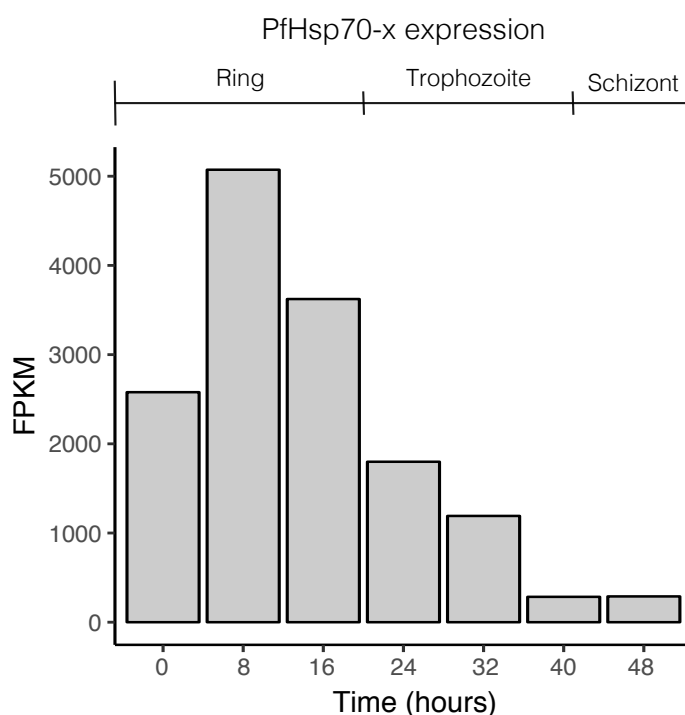


Figure 3.16: PfHsp70-x expression levels during intraerythrocytic development. Transcript levels of fragments per kilobase of exon model per million mapped reads (FPKM) vs hours in intraerythrocytic cycle. RNA collected at indicated time points post-infection from highly synchronised parasites. Data from [144].

We propose that the exported chaperone, and its partners, allow the translocation and trafficking of important parasitic proteins through the host cell cytoplasm. The delivery of parasitic proteins, such as pores for importing essential nutrients, to the iRBC surface may cause increased metabolism and thus cause the observed increase in growth. Additionally, it is likely that PfHsp70-x aids the export and delivery of virulence factors, such as PfEMP1, to the cell surface causing the increased cytoadherence previously reported. PfHsp70-x has been implicated in the export and presentation of PfEMP1 on numerous occasions due to their colocalization at certain time points and the presence of PfHsp70-x only in cytoadherent species

of *Plasmodia*. In fact, it was shown by Charnaud *et al* that their Δ PfHsp70-x parasites exported PfEMP1 less efficiently and iRBCs had reduced cytoadherence even without the proposed heat stress stimulus. PfHsp70-x's exact role in the proliferation of ring-stage parasites experiencing heat shock needs to be further studied by following protein export post-heat shock and in the absence of PfHsp70-x.

PfHsp70-x seems to also play a protective role in the trophozoite stage (24-36 hours) as seen from significantly reduced growth of the knockdown parasites in comparison to the control. Both cell lines show significantly reduced growth caused by the heat shock indicating this stage of the parasite development is more sensitive to stress compared to the ring stage. Although fever would typically not occur at this point of the parasite's life cycle, multiple asynchronous infections are possible resulting in more frequent febrile episodes. I propose that PfHsp70-x protects and refolds heat-damaged proteins in the host cell cytoplasm during these heat sensitive stages of the life cycle. The functions of Hsp70 chaperones including aggregation prevention, refolding of denatured proteins and targeting unsalvageable proteins for degradation have been extensively studied and demonstrated. It has also been shown that PfHsp70-x is a fully functional Hsp70 capable of these functions [85] which, alongside the reduced growth in the PfHsp70-x knockdown line in response to heat shock, confirm the role of this exported chaperone in the protection of proteins crucial to parasite survival under stress. A number of additional chaperones are present in the iRBC, intraparasitic and host derived, accounting for the remaining growth of the parasites demonstrating that PfHsp70-x is not the sole determinant of parasite survival in stress but part of a network likely comprising of parasite and human derived chaperones.

Work by Charnaud *et al* did not find any relationship between the presence of PfHsp70-x and reaction to heat shock applied in the ring stage. However, in their study, they call attention to changes in gene expression observed in their PfHsp70-x knockout. They proposed that the parasite had compensated for the loss of PfHsp70-x over the course of the months required for the knockout creation by altering expression patterns of exported protein genes. Here we have shown this is likely to be

true as our conditional knockdown system did indeed react differently to heat shock. The conditional knockdown system leads to significantly reduced levels of protein on a timescale that does not allow selection of compensating parasites in the population to occur and thus is able to observe a phenotype not previously observable.

In the coming chapters, we aim to structurally characterise PfHsp70-x and some of its binding partners to provide information for drug design and also to gain further insight into the workings of the exported chaperone network. We shall also test a number of known Hsp70 inhibitors against PfHsp70-x for the same aims.

4

PfHsp70-x

Contents

4.1	Crystal Structure of PfHsp70-x_{ATPase}	73
4.1.1	Comparison with human Hsp72	82
4.1.2	The crystal structure of PfHsp70-x _{ATPase} :ADP	85
4.2	HSQC NMR of the PfHsp70-x_{ATPase} nucleotide cycle	90
4.3	Discussion	95

4.1 Crystal Structure of PfHsp70-x_{ATPase}

We aim to explore the differences in structure between PfHsp70-x and human Hsp70s. Studying the structure of PfHsp70-x may give us an insight into its role, e.g. a different mechanism of action, as well as information that can be used to selectively inhibit the parasitic chaperone. In this thesis, we focus on the structure of the ATPase domain of PfHsp70-x as this is predicted to be the major binding site of many Hsp70 inhibitors, cochaperones and nucleotide exchange factors and thus more interesting to us.

Several attempts were made to crystallize the ATPase domain of PfHsp70-x by using different constructs, varying protein concentrations and co-crystallisation with ligands. Initially, crystal trials were set up using a construct containing both

the ATPase domain and linker of PfHsp70-x, however, this proved unsuccessful so a construct without the flexible linker was created: PfHsp70-x_{ATPase}. PfHsp70-x_{ATPase}, in the presence of AMP-PNP, a non-hydrolysable ATP analogue, crystallized and a resulting structure was solved to a resolution of 1.81 Å (Table 4.1).

Examination of the structure shows that the asymmetric unit consists of two nearly-identical copies (RMSD 0.293 Å) interacting through a small interface comprising of four salt bridges formed between residues R51 and D182 and two hydrogen bonds formed by N50 – Q186 and Y164 – D182 backbone atoms (Fig. 4.1). Protein Interfaces, Surfaces and Assemblies analysis (PISA) was used to assess the protein interaction interfaces for biological relevance relating to oligomeric state of the protein [145]. Two additional interfaces were found between symmetry-related copies however PISA predicted all interfaces to be artefacts of crystal packing on the basis of interface area, solvation and binding energies, hydrophobicity and the number of bonds involved in the interaction. Quality of NMR spectra shown later in this chapter is consistent with that of a 45 kDa protein and not that of a 90 kDa dimer confirming the inability of PfHsp70-x_{ATPase} to form dimers in solution.

PfHsp70-x_{ATPase} consists of a mixture of alpha helices and small beta sheets that form two lobes (I and II), each consisting of subdomains A and B, forming a channel between them that leads to the nucleotide binding site (Fig. 4.2). Within the nucleotide binding site, good density for AMP-PNP could be seen allowing easy placement of the ligand (Fig. 4.3).

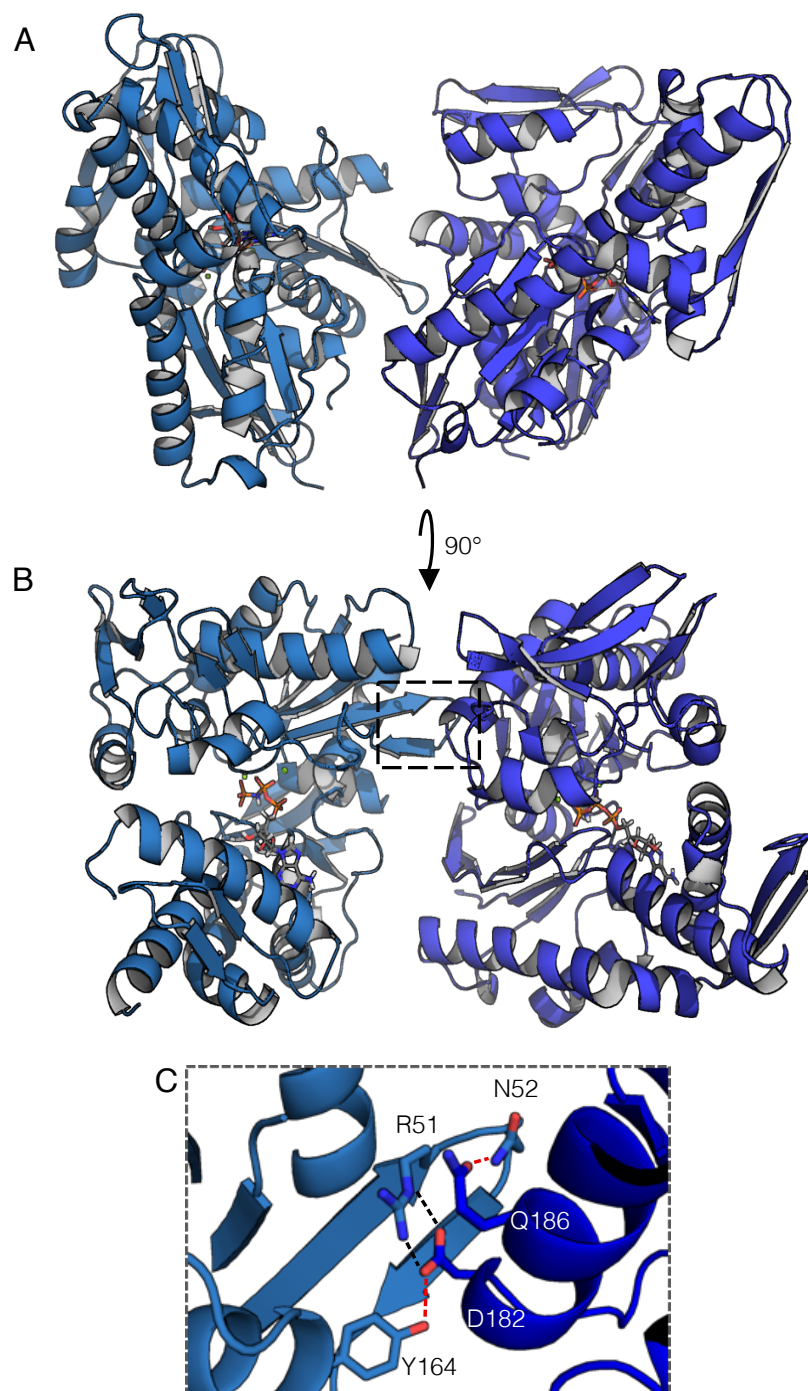


Figure 4.1: Asymmetric unit interactions of PfHsp70-x_{ATPase}:AMP-PNP. **A:** 'Side' view of the two copies, A (light blue) and B (blue), of PfHsp70-x_{ATPase}:AMP-PNP shown in cartoon representation. **B:** 'Top' view of the two copies, A (light blue) and B (blue), of PfHsp70-x_{ATPase}:AMP-PNP with the site of interaction highlighted by a dashed box. **C:** Closer view of the amino acids involved in the interaction interface. Salt bridges are shown by a black dotted line and are formed between D182 and R51. Hydrogen bonds are shown by dotted red lines and are formed between Y164 and D182 as well as N50 and Q186.

Table 4.1: PfHsp70-x_{ATPase}:AMP-PNP X-ray crystallography structure data collection and refinement statistics. Geometry and steric analysis was performed by the Molprobit server [146].

Protein	PfHsp70-x _{ATPase} AMP-PNP
Space group	P 2 ₁ 2 ₁ 2 ₁
Unit cell (Å,°)	80.29 102.71 103.77, 90 90 90
Beamline	DLS/I03
Wavelength (Å)	0.97625
Resolution range (Å)	43.26 – 1.81
High-resolution shell (Å)	1.84 – 1.81
R _{merge} (I)	0.057 (1.523)
R _{meas} (I)	0.062 (1.65)
R _{pim} (I)	0.024 (0.63)
Completeness (%)	99.9 (99.8)
Multiplicity	6.5 (6.7)
I/σ	12.65 (1.1)
CC _{1/2}	0.99 (0.72)
Wilson B factor	38.74
Refinement statistics	
R _{work} (reflections)	0.1869 (77,039)
R _{free} (reflections)	0.2097 (3,848)
Number of atoms	
Protein	5836
Ligands	66
Water	231
Average B factors (Å²)	
Protein	60.25
Ligands	45.73
Water	49.89
RMSD from ideal	
Bonds / angles (Å / °)	0.005 / 1.05
MolProbity statistics	
Ramachandran favored (%)	99.5
Ramachandran allowed (%)	0.54
Ramachandran outliers (%)	0
Rotamer outliers (%)	0
Clashscore (percentile)	1.44 (100 th)
MolProbity score (percentile)	0.88 (100 th)

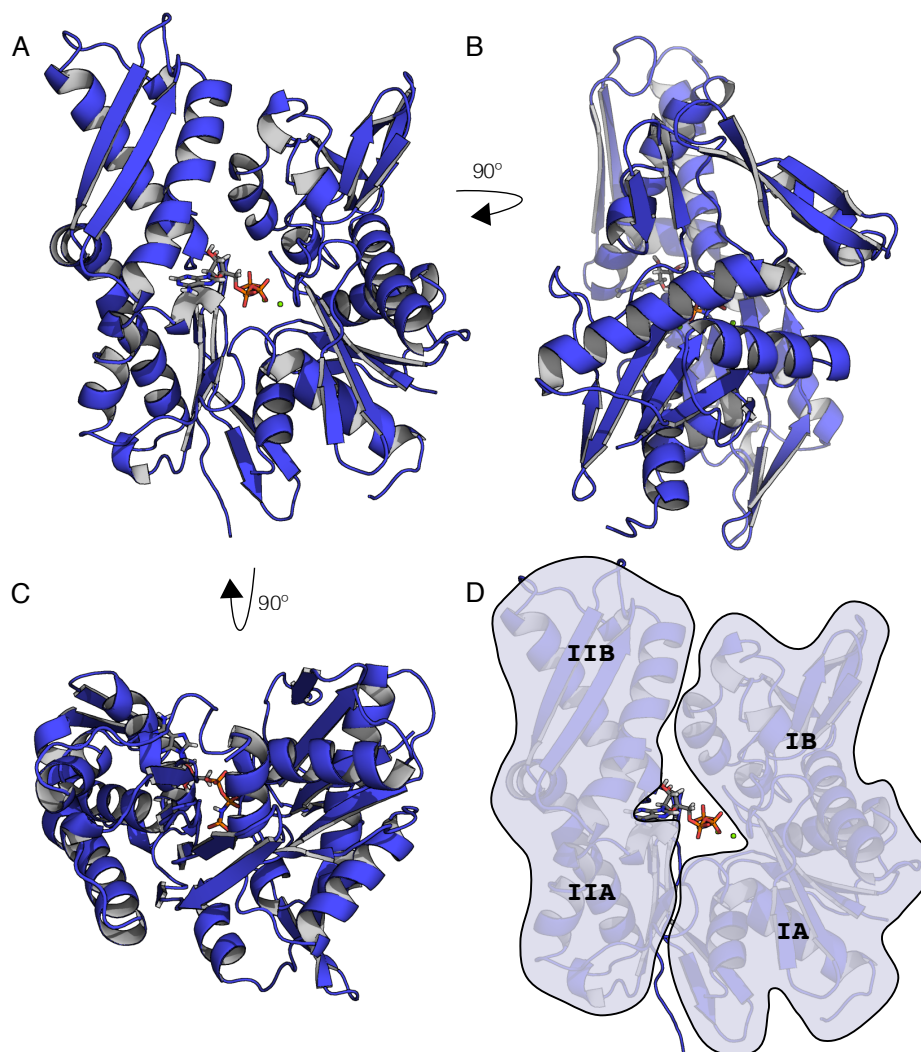


Figure 4.2: PfHsp70-x_{ATPase}:AMP-PNP crystal structure. **A:** 'Front' view of PfHsp70-x_{ATPase} with AMP-PNP (grey, coloured by element) and magnesium (green). **B:** Side view of PfHsp70-x_{ATPase}. **C:** Bottom view of PfHsp70-x_{ATPase}. **D:** The two lobes of PfHsp70-x_{ATPase} and the subdomains of each lobe.

Looking closer at the nucleotide binding site, we can see that there are many interactions between ligand and protein mostly mediated by phosphate groups of AMP-PNP and backbone amides of PfHsp70-x_{ATPase} (Fig. 4.3). There are also several interactions between the ligand and amino acid sidechains; specifically, T42 and T43, which form hydrogen bonds to the beta and gamma phosphate groups. The side chains of E299, K302 and S304 hydrogen bond to the adenosine moiety. In the context of the 3D structure (Fig. 4.4), we can also see how highly ordered water

molecules, alongside magnesium ions, form a more extensive network of interactions in the active site. The phosphate groups of AMP-PNP are able to form so many interactions with backbone amides as they are trapped by two glycine-rich loops: 230-240 and 40-60. The two loops that trap AMP-PNP's phosphates also trap a number of waters and magnesium ions forming a hydrophilic cavity. This highly hydrated cavity allows formation of many hydrogen bonds which position waters for nucleophilic attack on the nucleotide β - γ bond and stabilise the intermediate state therefore allowing for hydrolysis of the nucleotide.

Upon examination of the structure's refinement statistics (Table 4.1) we can see that the model features high B-factors when comparing to structures of similar resolution deposited in the PDB. When mapping the B-factors onto the surface of PfHsp70-x_{ATPase}, it becomes apparent that the high B-factors are localised to the extremities of the protein, particularly surface lysines and arginines, and to exposed loops (Fig. 4.5). The high flexibility of these residue side chains and disordered loops explain the high average B-factor and as the rest of the statistics reflect a good quality model, this is not considered an issue.

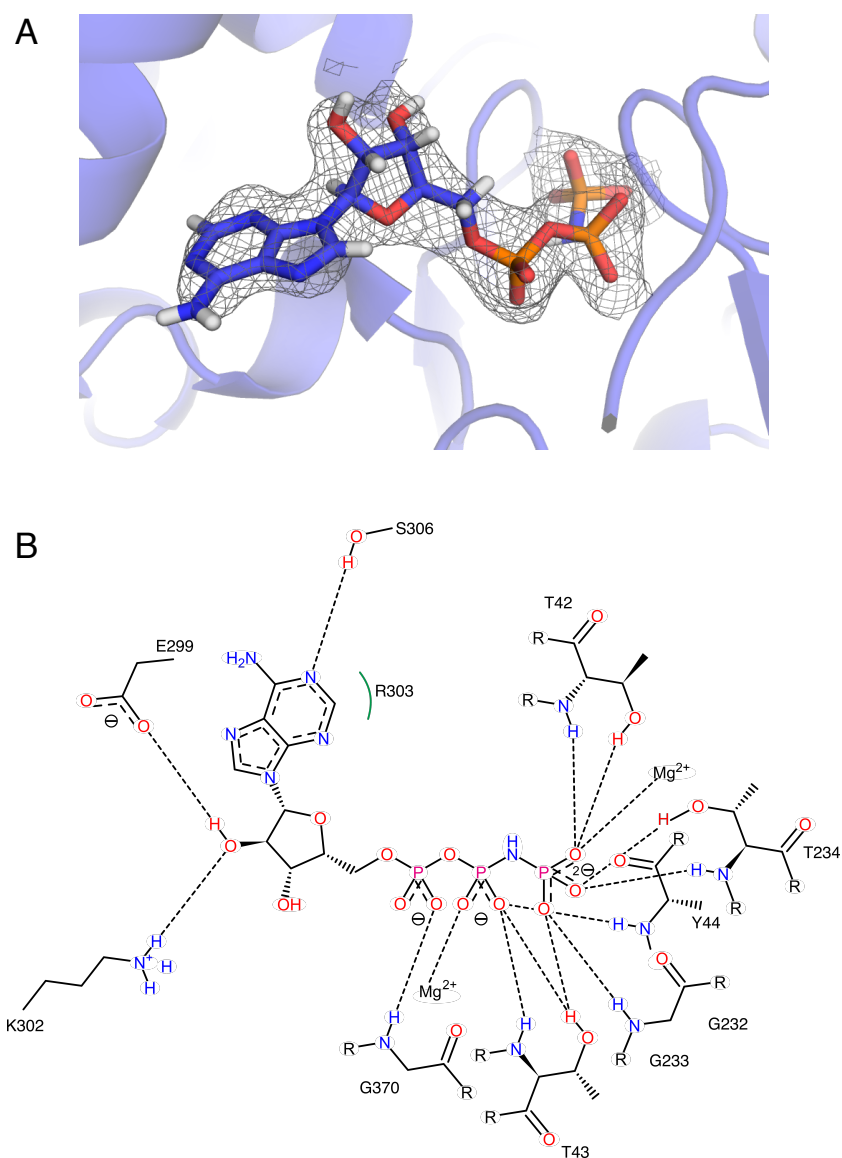


Figure 4.3: AMP-PNP ligand. **A:** Zoomed view of the AMP-PNP ligand within a composite omit 2mFo-DFc electron density map (grey mesh) at 3σ. **B:** 2D representation of AMP-PNP's interactions with PfHsp70-xATPase. Hydrogen bonds and charge interactions are represented by dashed lines and hydrophobic interactions by green arcs. Image generated by PoseView [147].

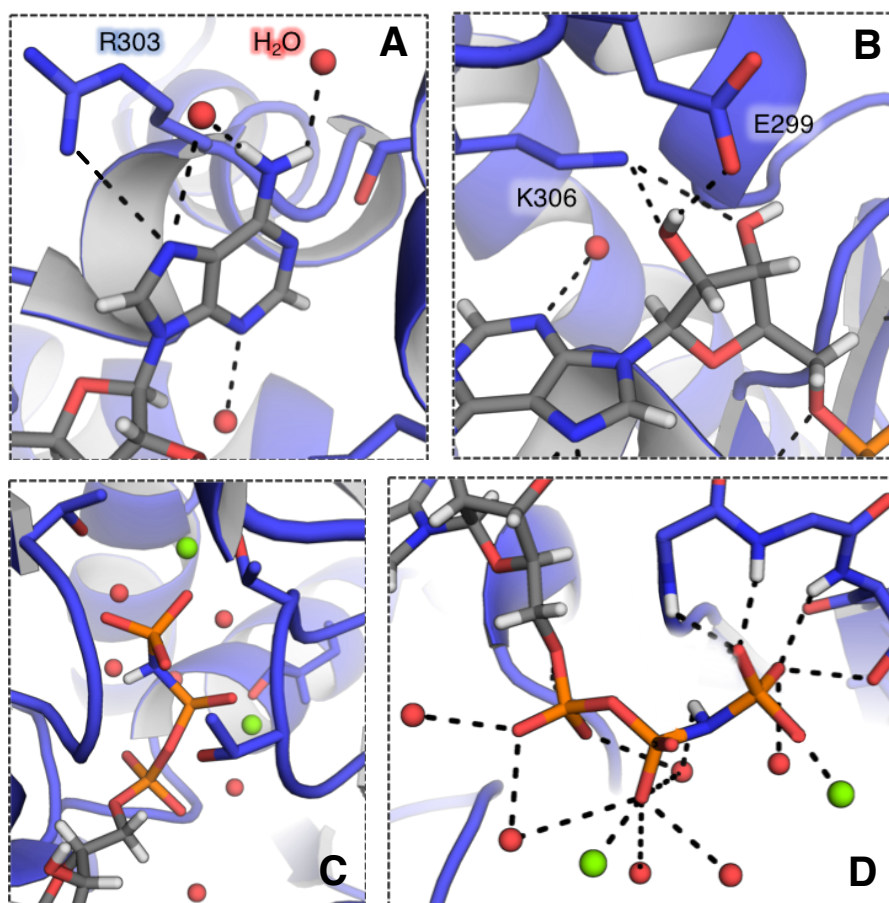


Figure 4.4: 3D examination of AMP-PNP ligand interactions. **A:** Interactions of the adenine moiety of AMP-PNP. **B:** Interactions of the ribose moiety of AMP-PNP. **C:** Phosphate tail of AMP-PNP trapped in hydrated cavity by two loops. **D:** A closer look at the interactions of the phosphates of AMP-PNP with loop residues, ordered waters (red) and magnesium ions (green).

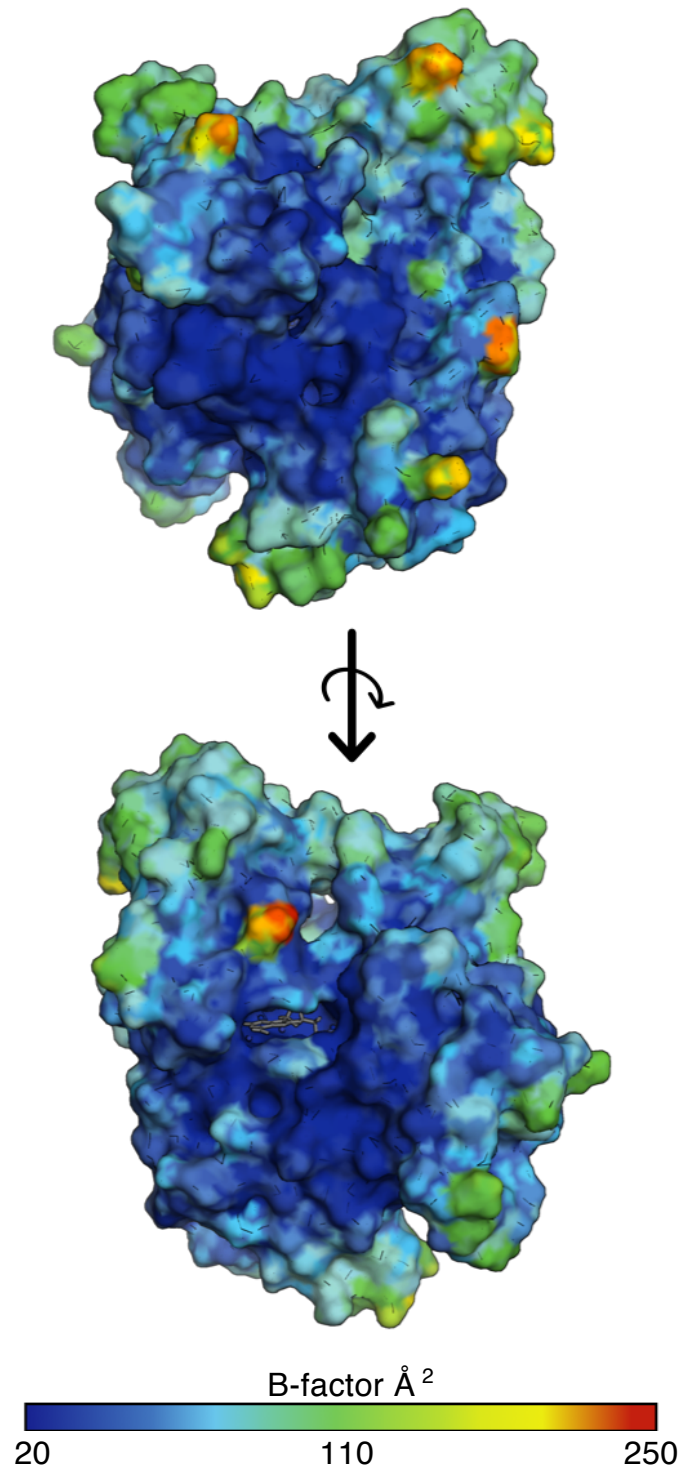


Figure 4.5: B-factors of PfHsp70-xATPase:AMP-PNP Structure of the PfHsp70-xATPase:AMP-PNP structure coloured by B-factor per atom shown by surface representation.

4.1.1 Comparison with human Hsp72

PfHsp70-x_{ATPase} has a canonical Hsp70 ATPase domain fold consisting of two lobes each made up from two subdomains as previously described. This structure is seen across all domains of life as seen when comparing the ATPase domain PfHsp70-x, human Hsp72 [148], bacterial DnaK [149] and yeast Bip [150] in Figure 4.6.

The all-atom RMSD between PfHsp70-x and Hsp72 demonstrates that the structures are very similar with a value of 0.7 Å. A pairwise alignment of PfHsp70-x and Hsp72 shows that the proteins share 67% identity and 80% similarity. By mapping the residues that differ onto the aligned structures, we can see non-conserved patches indicating differences that can be targeted in drug design (Fig. 4.7). Immediately, we can see that the active site is highly conserved and the active site of the structures overlay very well. Therefore, we can infer that it is unlikely a nucleotide mimic would have the ability to specifically inhibit PfHsp70-x. Outside the active site there are many patches of non-conserved residues on all subdomains which are localised largely to the protein surface. Three patches of non-conserved residues are shown indicating possible sites to target with small molecules that may allosterically inhibit PfHsp70-x rather than directly inhibiting ATP binding/hydrolysis (Fig. 4.8).

One option for allosterically inhibiting PfHsp70-x via the ATPase domain involves preventing the rotation of subdomains in relation to each other which is caused by SBD or J domain docking as well as nucleotide exchange or hydrolysis. This rotation of subdomain has been reported to optimally align the catalytic residues in addition to opening the SBD for substrate binding. A second option would be to directly prevent docking of the SBD or Hsp40 J domain through the design of a small molecule that competes with such domains.

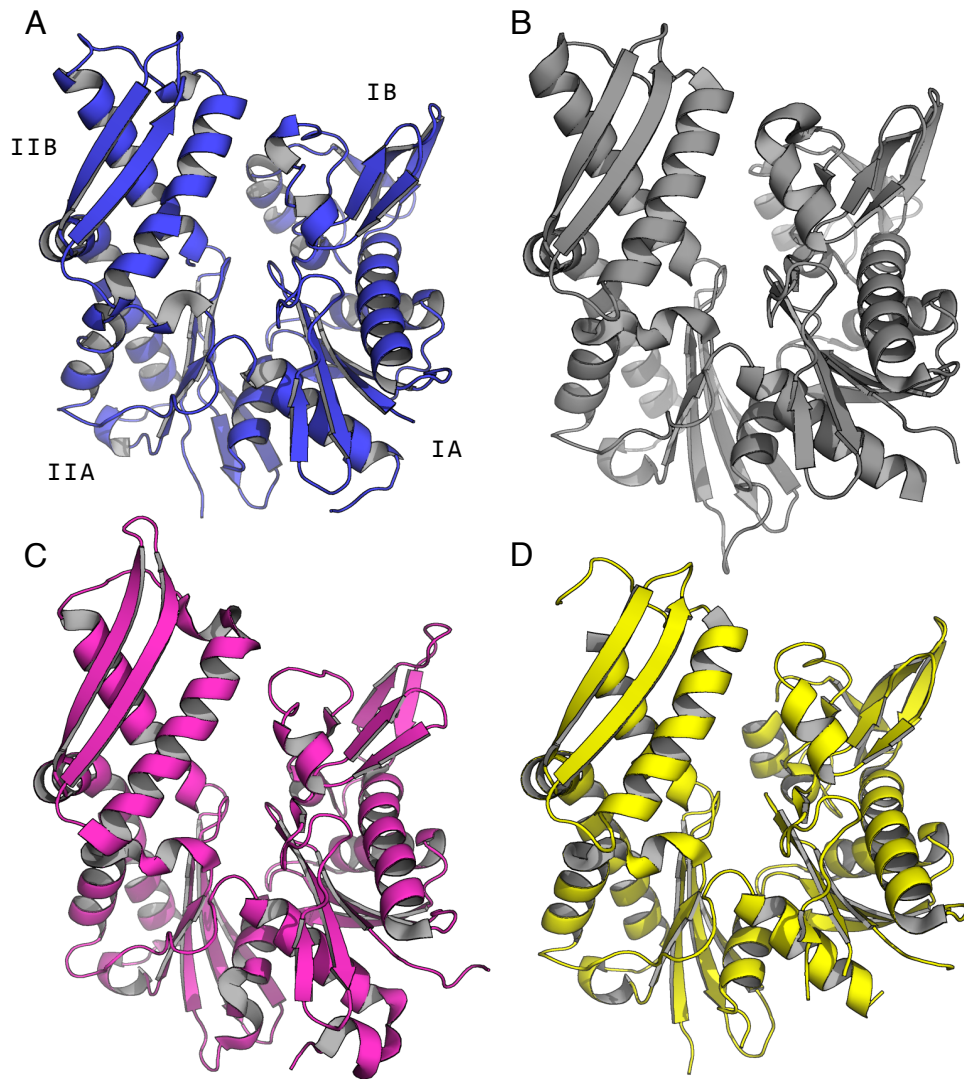


Figure 4.6: PfHsp70-x has the canonical Hsp70_{ATPase} architecture. **A:** PfHsp70-x_{ATPase} structure. **B:** human Hsp72 ATPase domain structure (PDB 2E8A [148]). **C:** yeast Bip ATPase domain structure (PDB 3QFP [150]). **D:** *Mycoplasma genitalium* DnaK ATPase domain structure (PDB 5OBY [149]).

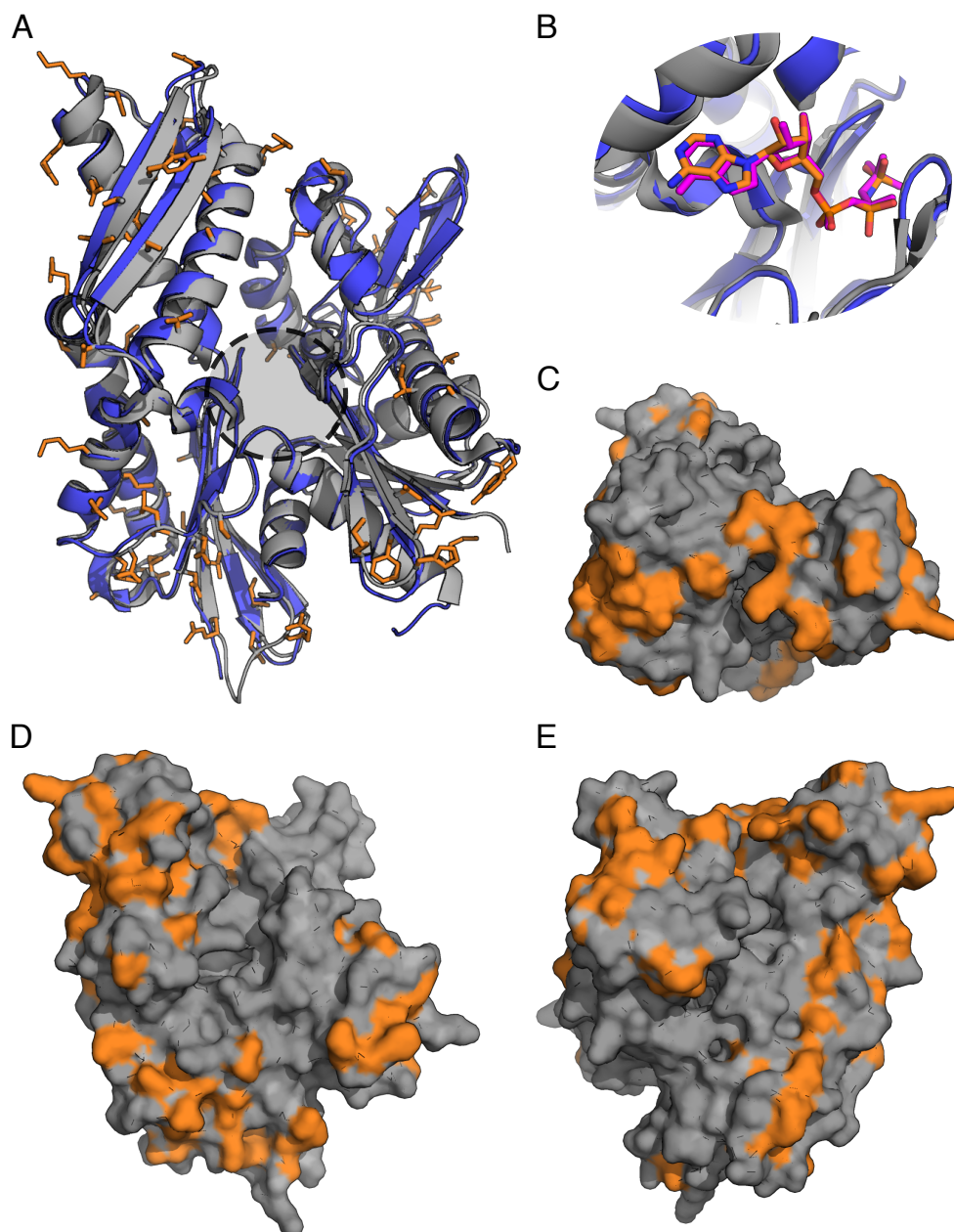


Figure 4.7: Comparison of PfHsp70-x and human Hsp72 **A:** Aligned PfHsp70-x_{ATPase} and human Hsp72_{ATPase} structures. Residues that differ between the proteins have their side chains shown by stick representation and coloured orange. **B:** Close-up of the active site of A showing the overlap of AMP-PNP from the PfHsp70-x structure (orange carbons) and the Hsp72 structure (magenta carbons). AMP-PNP is shown in stick representation with atom coloring as previously described). **C:** Surface representation showing the localisation of non-conserved residues (orange) shown from below. **D:** Surface representation showing the localisation of non-conserved residues (orange) shown from the front. **E:** Surface representation showing the localisation of non-conserved residues (orange) shown from behind.

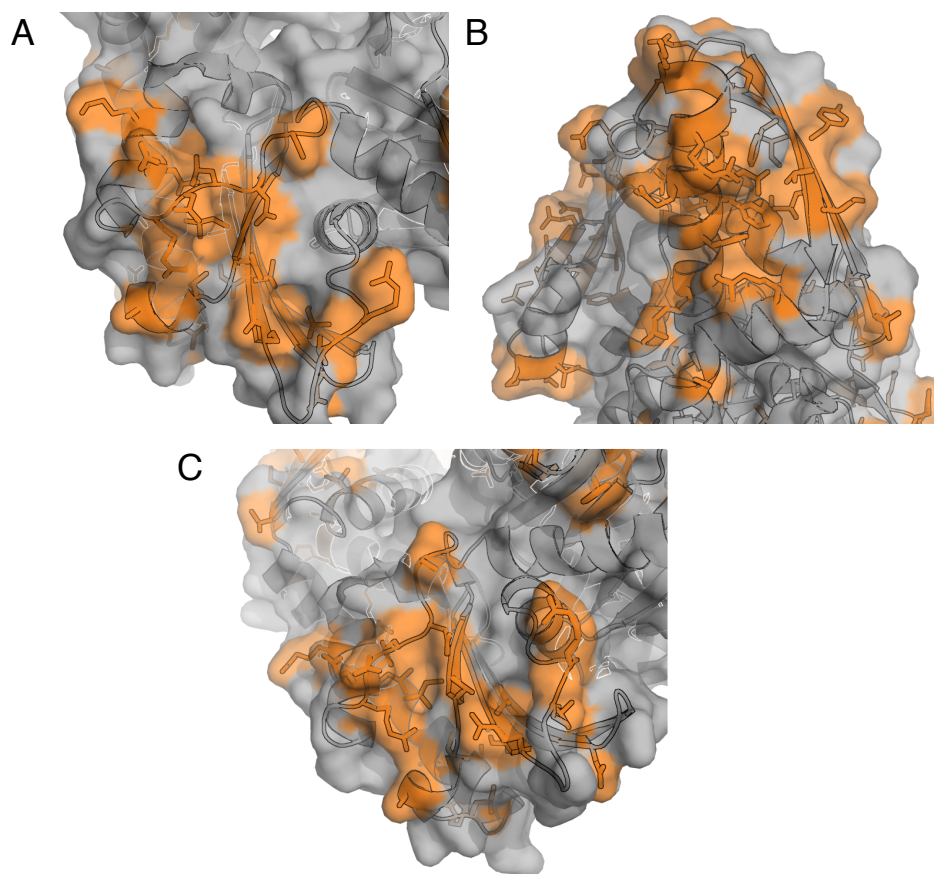


Figure 4.8: Non-conserved patches of PfHsp70-x_{ATPase} **A:** Close-up of non-conserved patch I. **B:** Close-up of non-conserved patch II **C:** Close-up of non-conserved patch III.

4.1.2 The crystal structure of PfHsp70-x_{ATPase}:ADP

Crystals were also obtained by cocrystallizing PfHsp70-x_{ATPase} with ADP, the product of ATP hydrolysis and the structure solved to a resolution of 1.87 Å. PfHsp70-x_{ATPase} in complex with ADP crystallised in different conditions compared to the complex with AMP-PNP, but the crystals had near identical unit cell dimensions and the same space group (Table 4.2).

It was expected that the AMP-PNP- and ADP-bound structures would have different conformations representing different stages of the nucleotide cycle. However, upon comparing structures, we observe that the AMP-PNP and ADP bound forms are extremely similar with an all-atom RMSD of 0.33 Å when aligned (Fig. 4.9).

No significant conformational changes can be seen, and the majority of differences localise to loops that display poorer density and higher B-factors suggesting inherent flexibility. The density of the ligand in the active site fits ADP very well confirming that there is no ATP carried over from purification (Fig. 4.10).

Looking closely at the active site, we see that the ADP and AMP-PNP nucleotides overlay extremely well especially at the adenine and ribose moieties (Fig. 4.10). No significant backbone or side chain movements can be seen within the active site.

Table 4.2: PfHsp70-x_{ATPase}:ADP X-ray crystallography structure data collection and refinement statistics.

Protein	PfHsp70-x _{ATPase} ADP
Space group	P 2 ₁ 2 ₁ 2 ₁
Unit cell (Å,°)	80.12 101.8 103.31, 90 90 90
Beamline	DLS/I04
Wavelength (Å)	0.9795
Resolution range (Å)	72.51 – 1.87
High-resolution shell (Å)	1.94 – 1.87
R _{merge} (I)	0.078 (1.67)
R _{meas} (I)	0.086 (1.82)
R _{pim} (I)	0.034 (0.72)
Completeness (%)	98.78 (94.87)
Multiplicity	6.3 (6.3)
I/σ	10.33 (1.10)
CC _{1/2}	0.998 (0.582)
Wilson B factor	30.99
Refinement statistics	
R _{work} (reflections)	0.2224 (69,587)
R _{free} (reflections)	0.2518 (3386)
Number of atoms	
Protein	5770
Ligands	56
Water	287
Average B factors (Å²)	
Protein	48.1
Ligands	38.0
Water	40.1
RMSD from ideal	
Bonds / angles (Å / °)	0.007 / 1.07
MolProbity statistics	
Ramachandran favored (%)	97.6
Ramachandran allowed (%)	2.4
Ramachandran outliers (%)	0
Rotamer outliers (%)	0.32
Clashscore (percentile)	2.4 (99 th)
MolProbity score (percentile)	1.11 (100 th)

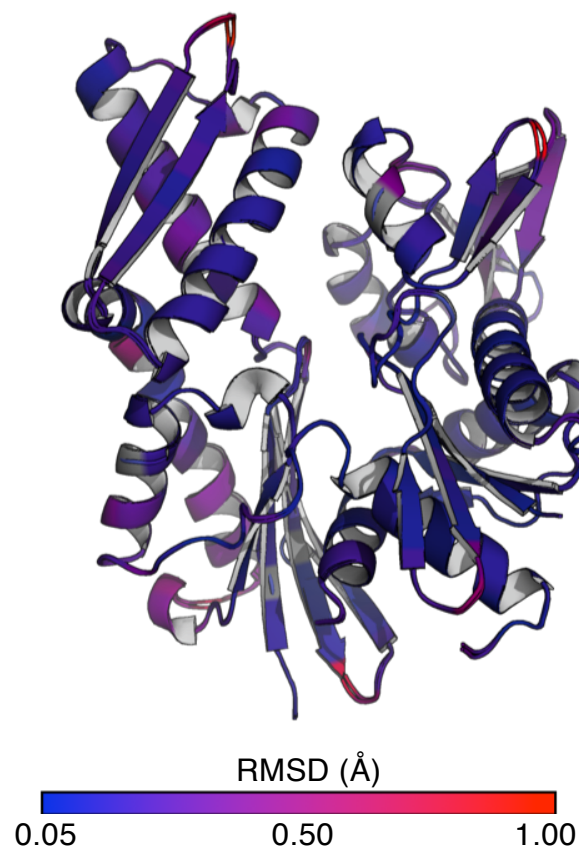


Figure 4.9: Aligned PfHsp70-x_{ATPase}:AMP-PNP and PfHsp70-x_{ATPase}:ADP structures coloured by RMSD.

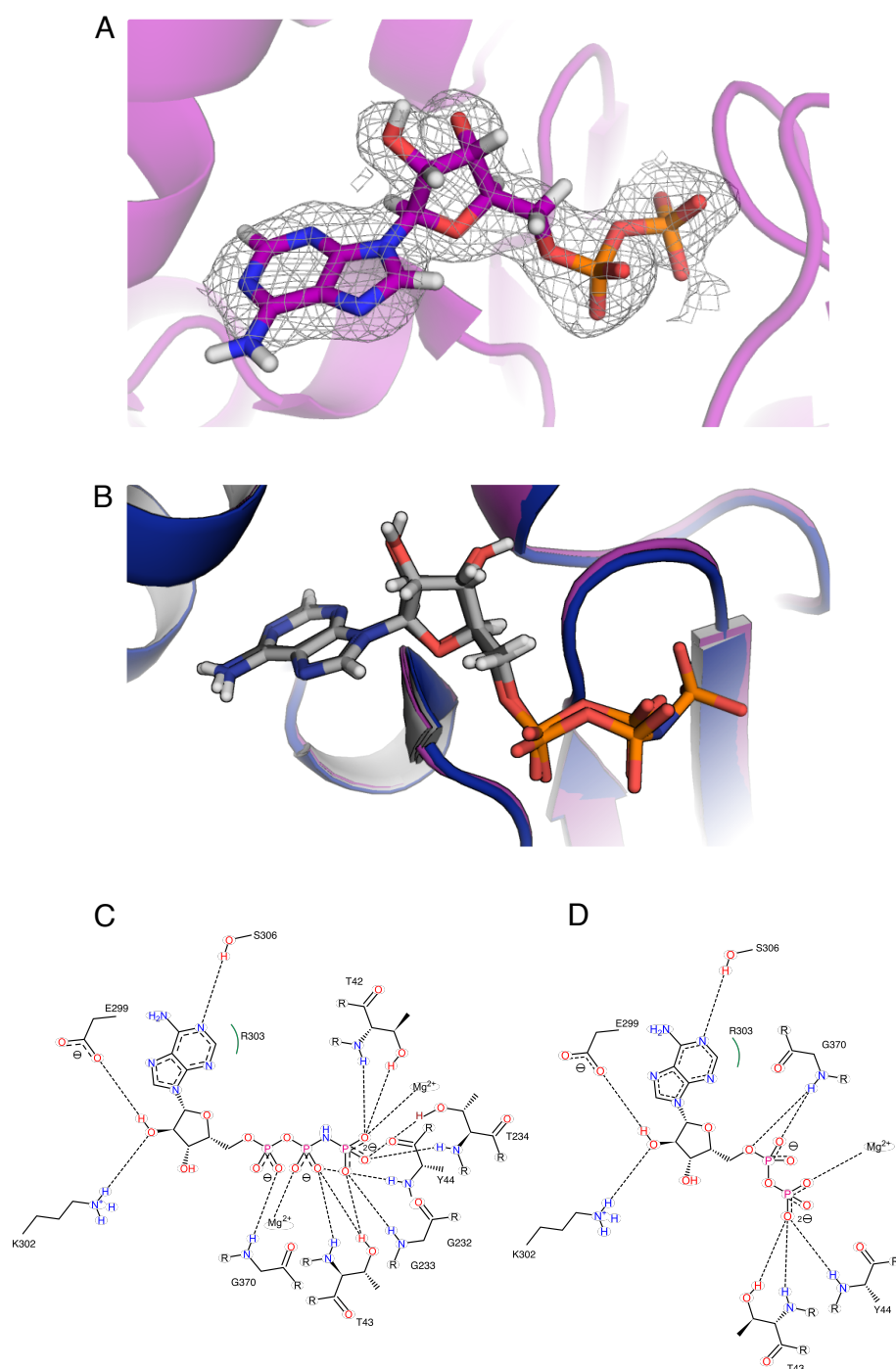


Figure 4.10: Comparison of PfHsp70-xATPase:ADP and PfHsp70-xATPase:AMP-PNP ligand binding. **A:** Zoomed view of the ADP ligand within a composite omit 2mFo-DFc electron density map (grey mesh) at 3σ. **B:** Alignment of the PfHsp70-xATPase structures focusing on the nucleotide binding site demonstrating the identical poses of the ligands. **C:** 2D representation of AMP-PNP interactions generated by PoseView. **D:** 2D representation of ADP interactions generated by PoseView.

4.2 HSQC NMR of the PfHsp70-xATPase nucleotide cycle

To further explore if the PfHsp70-x nucleotide binding domain changes conformation throughout the nucleotide cycle, nuclear magnetic resonance (NMR) ^1H - ^{15}N heteronuclear single quantum coherence (HSQC) was employed. HSQC pulse sequences first excite protons in the protein followed by magnetisation transfer to ^{15}N via covalent bonds, and then back to proton for detection as a free induction decay. The resultant HSQC spectra are made up of peaks representing one amino acid. The resonance frequency of each nucleus depends on its chemical environment and therefore can be used to follow changes of environment such as conformational changes, protein-protein or protein-ligand interactions and chemical reactions, and therefore ^1H - ^{15}N HSQC is used extensively through this thesis.

By overlaying PfHsp70-xATPase spectra in the absence of nucleotides (apo), with spectra in the presence of AMP-PNP or ADP, it is possible to study potential conformational changes involved in the nucleotide cycle (Fig. 4.11). AMP-PNP is a non-hydrolysable ATP analogue that can trap the ATPase domain in the ATP bound state and was used in place of mutating the catalytic residues to preserve the native structure. Compared to the apo state, the AMP-PNP bound state exhibits many chemical shift perturbations mostly in the form of peak changes in position. We can see that the majority of peaks change position when comparing AMP-PNP and apo states suggesting the majority of amino acids have an altered chemical environment which is likely caused by a conformational change in the ATPase domain structure. When comparing ADP-bound and apo spectra, we also see a large number of peaks changing position suggesting a conformational change upon ADP binding, too. Interestingly, when comparing the ADP-bound spectra with those in the presence of AMP-PNP we see few peak changes (Fig. 4.12), suggesting that the ADP- and AMP-PNP-bound states are similar.

Hsp70s require magnesium for ATP hydrolysis as a magnesium ion positions the ligand and important waters in the correct conformation for hydrolysis. The addition of magnesium to the nucleotide cycle was also studied to understand

if magnesium affects the conformational changes seen upon addition of ADP or AMP-PNP (Fig. 4.13). We see additional peak shifts particularly for the ADP- Mg^{2+} bound state resulting in large differences between the AMP-PNP Mg^{2+} and ADP Mg^{2+} bound states. It is possible that the presence of magnesium alters the affinity of nucleotides binding and so rather than conformational changes, perhaps we are simply viewing the equilibrium of nucleotide-free and -bound states being shifted towards more nucleotide-bound protein. This logic can be extended to the effect of magnesium addition being larger on ADP than AMP-PNP; AMP-PNP is able to hydrogen bond more extensively through its additional phosphate moiety and thus often has a stronger affinity than ADP. Therefore, at equivalent molar ratios, there will be more unbound nucleotide in the ADP system rather than in the AMP-PNP system resulting in magnesium having a more noticeable effect of the equilibrium of bound and unbound ADP.

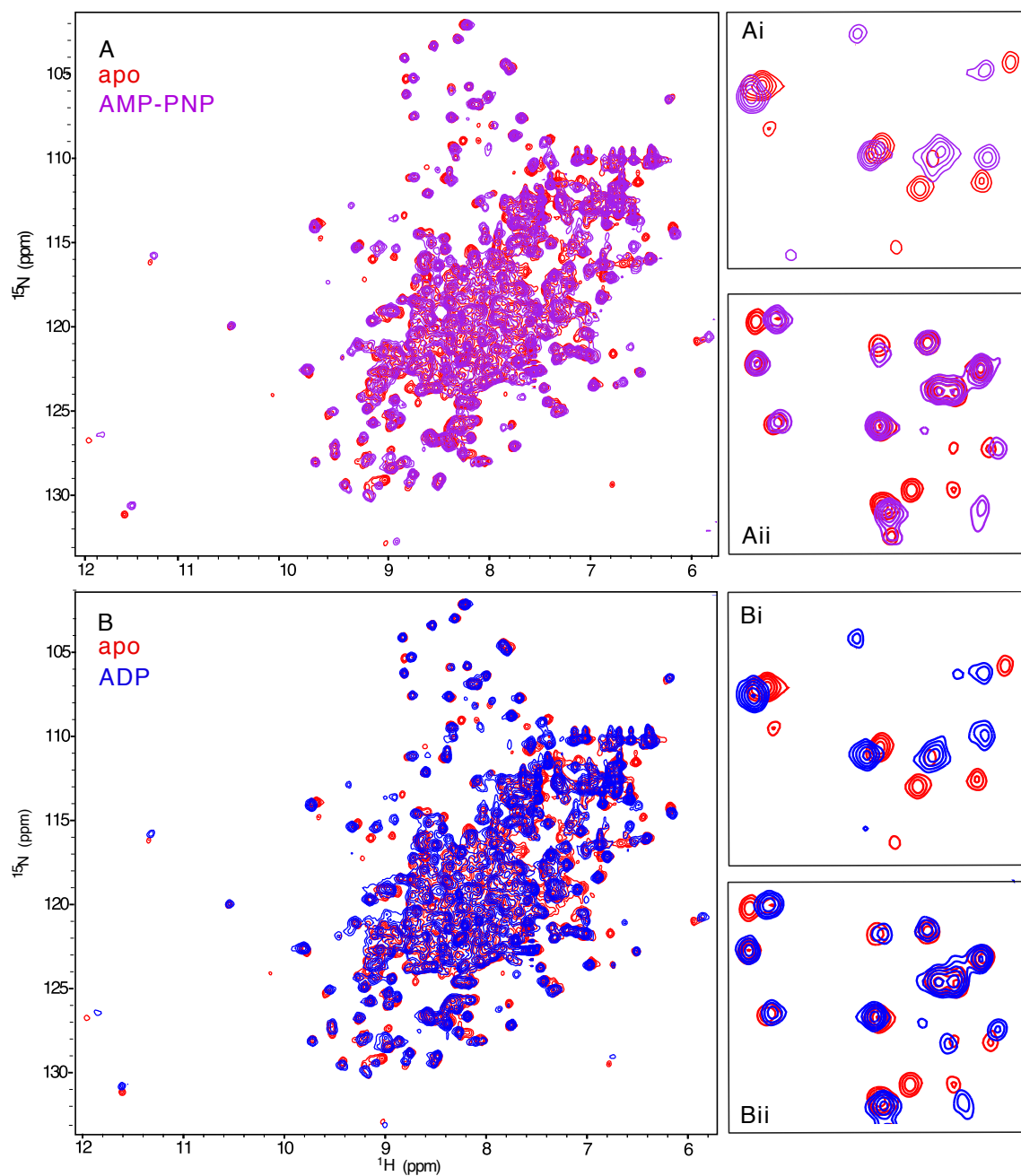


Figure 4.11: ^1H - ^{15}N HSQC spectra of *PfHsp70-x*ATPase with nucleotides. **A:** Overlay of apo *PfHsp70-x*ATPase (red) and *PfHsp70-x*ATPase:AMP-PNP spectra (purple). **B:** Overlay of apo *PfHsp70-x*ATPase (red) and *PfHsp70-x*ATPase:ADP spectra (blue).

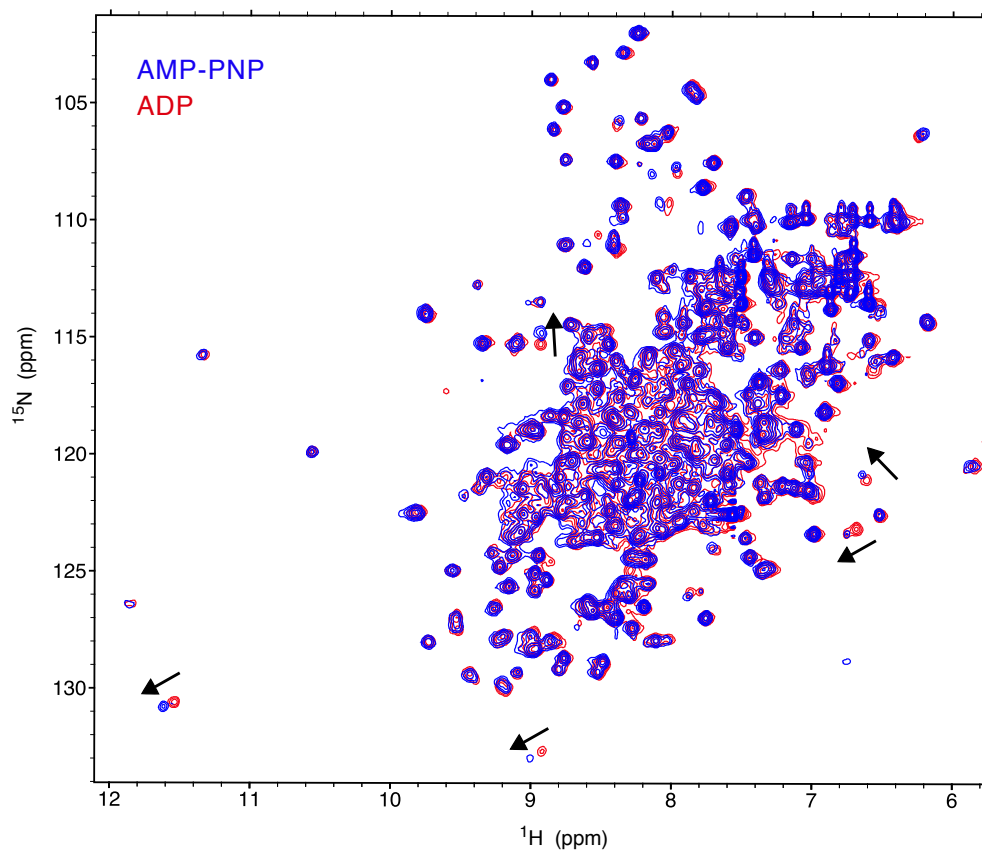


Figure 4.12: ^1H - ^{15}N HSQC spectra of PfHsp70-xATPase with ADP and AMP-PNP. Overlay of $\text{PfHsp70-xATPase:ADP}$ (red) and $\text{PfHsp70-xATPase:AMP-PNP}$ spectra (blue). Arrows indicate large peak shifts and their direction from ADP to AMP-PNP states.

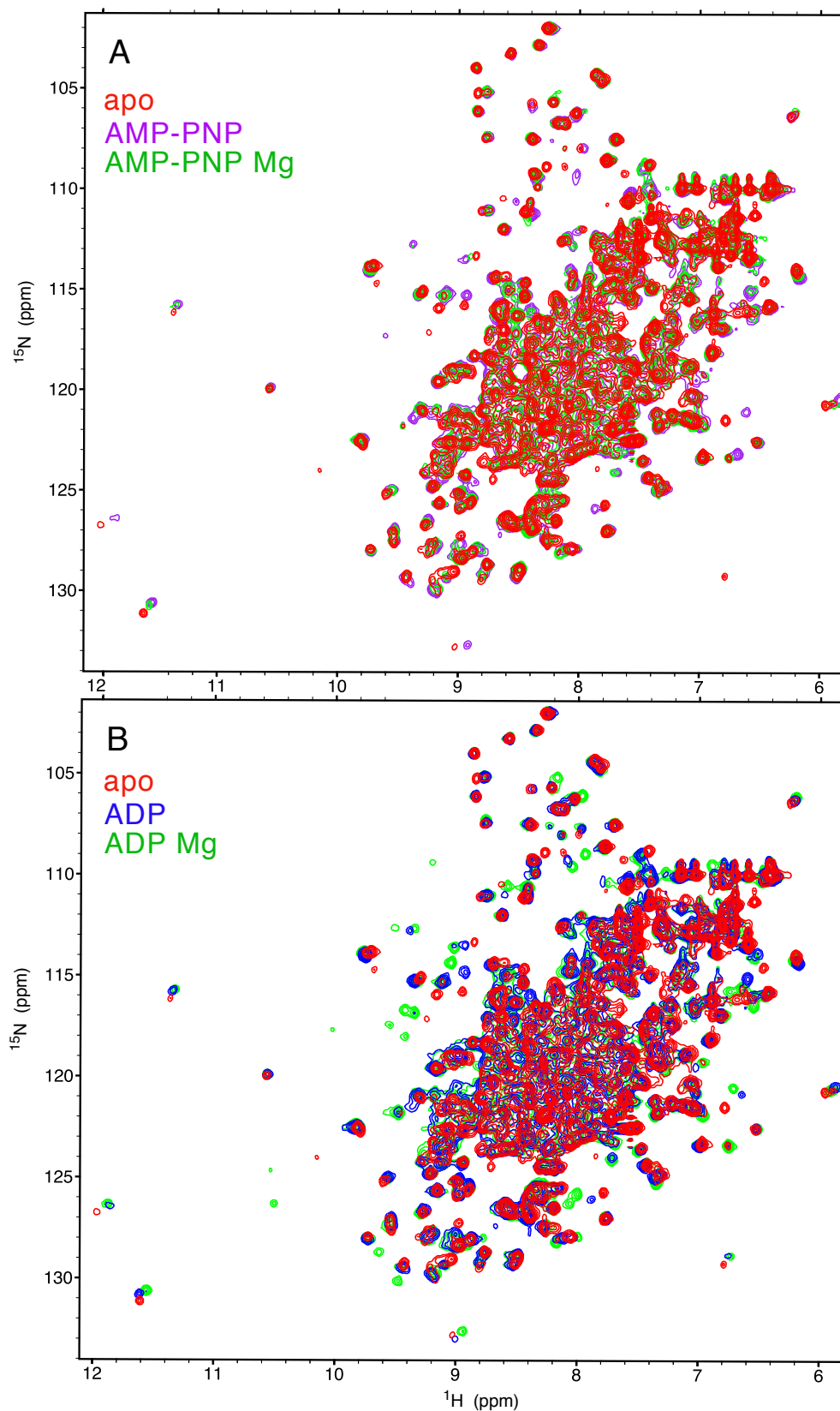


Figure 4.13: The effect of Mg^{2+} on ATPase domain structure. **A:** Overlay of apo PfHsp70-xATPase (red), PfHsp70-xATPase:AMP-PNP spectra (purple) and PfHsp70-xATPase:AMP-PNP + Mg^{2+} spectra (green). **B:** Overlay of apo PfHsp70-xATPase (red), PfHsp70-xATPase:ADP spectra (blue) and PfHsp70-xATPase:ADP + Mg^{2+} spectra (green).

4.3 Discussion

In this chapter, we presented the crystal structure of PfHsp70-x_{ATPase} in complex with AMP-PNP. By studying the structure and comparing with human homologues, we were able to identify regions of poor conservation that may be used to design specific drugs against PfHsp70-x. Additionally, we could see that the active site is highly conserved and binds nucleotides through the same mechanism ruling out ATP mimetics as a means to specifically drug PfHsp70-x.

During crystallisation trials, crystals were also successfully grown in the presence of ADP. Interestingly, the structures of AMP-PNP and ADP bound PfHsp70-x_{ATPase} were nearly identical which is perhaps unexpected. ATP hydrolysis, and the associated conformational changes, are integral to the chaperone activity of Hsp70s [39, 151, 152]. The ATPase domain, the site of ATP hydrolysis, of Hsp70 proteins is tethered loosely to the substrate binding domain (SBD) by a hydrophobic and flexible linker, suggesting that the ATPase domain acts in isolation [32]. The nucleotide state of the ATPase domain influences the SBD by influencing the inter-domain interaction affinities [38]. We would therefore anticipate that looking at the ATPase domain in isolation may still allow us to study the conformational changes in different nucleotide states. However, this is not the case with the presented PfHsp70-x_{ATPase} structures and, in fact, also in the case of previously published structures of the ATPase domain of human Hsp70 chaperones [153, 33, 154, 148]. Crystal structures of AMP-PNP and ADP bound forms of the isolated ATPase from both human and bovine Hsp70s show no discernible structural differences relating to nucleotide state. Additionally, small-angle X-ray scattering studies were not able to discern any difference between nucleotide bound states of isolated bovine Hsp70_{ATPase} [40].

It is only when looking to Hsp70 ATPase domains bound to nucleotide exchange factors (NEF) that it is possible to see what is denoted as the 'open' conformation [152, 155, 156, 157]. The open conformation shows a rotation of subdomain IIB and of subdomains IA and IIA in relation to each other. These subdomain rotations open the channel leading to the nucleotide binding pocket and disrupt interactions of the protein with the phosphates of ADP which allow the nucleotide to dissociate.

However, studies utilising NMR [158, 41, 159, 160], tryptophan fluorescence [161] and molecular dynamics (MD) simulations [162] demonstrated that the isolated ATPase domain exhibits greater flexibility than is seen in the crystal structures. Their findings show that the isolated ATPase domain in the ADP state closely represents the open conformation seen upon NEF binding. Moreover, this work indicates that the ATP bound ATPase domain would be even more tightly closed than is seen in the crystal structures. This tightly closed structure was indeed seen in the ATP-bound structures of full-length DnaK [50] and BiP [163] (*E. coli* and human Hsp70s respectively) in which the subdomains have independently rotated allowing the exposure of a suitable surface for SBD and linker docking and therefore stabilising the ATP-bound conformation.

We utilised NMR to further study the PfHsp70-x_{ATPase} nucleotide cycle and explore if there are conformational changes in solution as shown previously for other Hsp70s. We see many peaks changing position when comparing apo PfHsp70-x_{ATPase} to ADP and AMP-PNP bound states indicating a conformational change. However, upon comparing ADP and AMP-PNP bound PfHsp70-x_{ATPase} spectra we see very few changes in peak position indicating no significant changes in structure. It is only upon addition of magnesium that we see the further shifting of peaks in the ADP-bound spectrum. Therefore, we can hypothesise that magnesium results in a shift in equilibrium between the ADP- and AMP-PNP-bound conformations.

The structures presented in this chapter and those published previously of the isolated ATPase domain represent an intermediate conformation that is neither the ADP- nor ATP-bound conformation but is instead one that crystallizes readily. However, it must be remembered that proteins exist in an equilibrium of conformations and that, upon binding a particular nucleotide, the protein's equilibrium is shifted. X-ray crystallography provides us with snapshots along this spectrum of conformations and can also induce non-physiological structures by crystal packing. These structures are nevertheless important and useful particularly when used alongside techniques such as NMR and biophysical methods.

We can conclude from this chapter that PfHsp70-x has ATPase activity which induces some conformational change in the ATPase domain in solution but that cannot be observed through crystallography. The overall fold of PfHsp70-x_{ATPase} is identical to that of the human Hsp70_{ATPase} which is perhaps unsurprising as the overall structure of Hsp70s is highly conserved through all domains of life [164, 165, 166]. However, details of the structure will be useful in understanding the differences that allow for specific inhibition by small molecules and specific stimulation by cochaperones.

5

Inhibiting PfHsp70-x

Contents

5.1	The ATPase activity of PfHsp70-x	98
5.2	Testing Hsp70 inhibitors for PfHsp70-x inhibition . . .	102
5.3	The mechanism of inhibition	110
5.4	Studying ligand binding with STD-NMR	113
5.5	Discussion	118

Hsp70 inhibitors have been developed to treat cancer and neurodegenerative diseases. In this chapter, we aim to explore the potential of repurposing existing Hsp70 inhibitors as antimalarials. To do so, we establish an assay to measure the ATP hydrolysis activity of PfHsp70-x and test a number of inhibitors. Inhibition is then studied in greater detail through structural techniques and biophysics to gain insights into the mechanism of inhibition.

5.1 The ATPase activity of PfHsp70-x

A coupled enzymatic reaction assay was used to follow the hydrolysis of ATP by PfHsp70-x_{FL} over time. The assay uses purine nucleoside phosphorylase (PNPase) and free phosphate released from ATP hydrolysis to catalyse the formation of 2-amino-6-mercapto-7-methylpurine from 2-amino-6-mercapto-7-methylpurine riboside

(MESG) which has an increased absorbance at 360 nm [109]. Various inorganic phosphate sample concentrations were used to create a calibration curve to allow the conversion of absorbance to moles of ATP hydrolysed (Fig. 5.1). The MESG assay was optimized for PfHsp70-x by varying concentrations of the substrate of the coupled enzymatic reaction (MESG) (Fig. 5.2). 400 μ M MESG was chosen as the optimal concentration of MESG as this gave a strong UV signal with smaller errors without using large amounts of material. Upon testing a series of ATP concentrations to determine the kinetics of PfHsp70-x's ATP hydrolysis activity and to optimise the assay, we did not see typical Michaelis-Menten kinetics; rather, at high levels, ATP appeared to inhibit the coupled enzymatic assay. Thus, the ATP concentration for hydrolysis assays was set to 1 mM, as this produced strong hydrolysis without leading to strong inhibition. At this concentration of substrate, PfHsp70-x in our assay has an initial velocity (V_0) of 0.641 nmoles min^{-1} . As the kinetics do not follow the Michaelis-Menten model, we can only estimate the turnover number (K_{cat}) by assuming that the maximum velocity (V_{max}) is the highest initial velocity seen: 0.688 nmoles min^{-1} . We can then calculate the estimated K_{cat} by the following equation:

$$K_{\text{cat}} = \frac{V_{\text{max}}}{E_{\text{t}}} \quad (5.1)$$

In which K_{cat} is the turnover number, V_{max} is the maximum initial velocity and E_{t} is the concentration of enzyme active sites. The concentration of enzyme active sites was determined using a NanoDrop spectrophotometer to measure absorbance at 280 nm.

The K_{cat} of PfHsp70-x is $\sim 0.04 \text{ s}^{-1}$ which is considered slow for an enzyme. However, this is characteristic of Hsp70s as they require stimulation by cochaperones and nucleotide exchange factors to function at a higher rate [167, 168, 169].

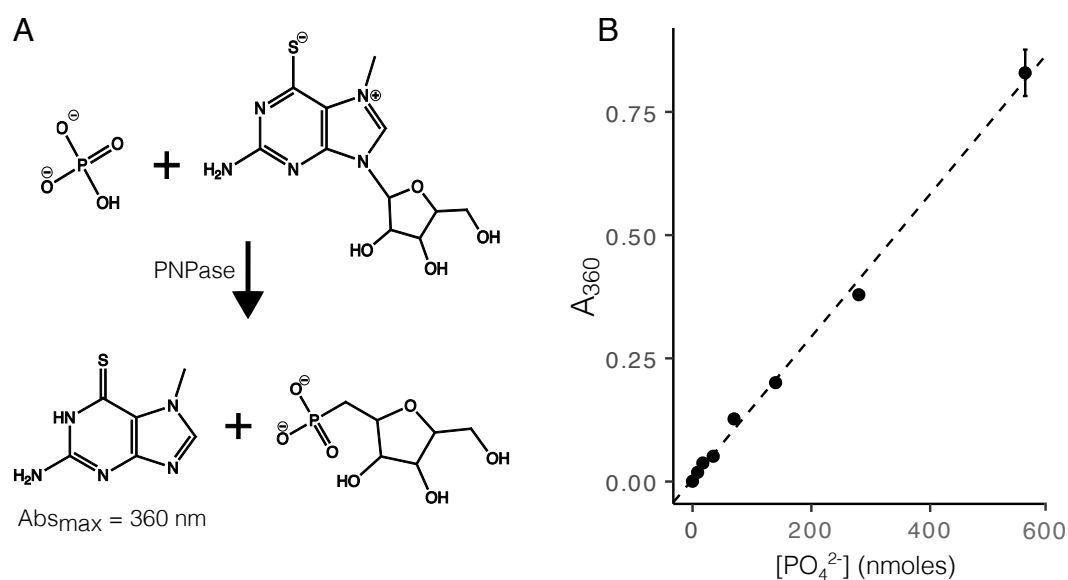


Figure 5.1: A continuous assay using the enzyme catalyzed breakdown of MESG to measure ATP hydrolysis. A: Conversion of MESG into a 2-amino-6-mercapto-7-methylpurine with absorbance maximum at 360 nm by PNPase and free phosphate. **B:** Calibration curve to convert absorbance at 360 nm to concentration of inorganic phosphate and therefore ATP hydrolysed.

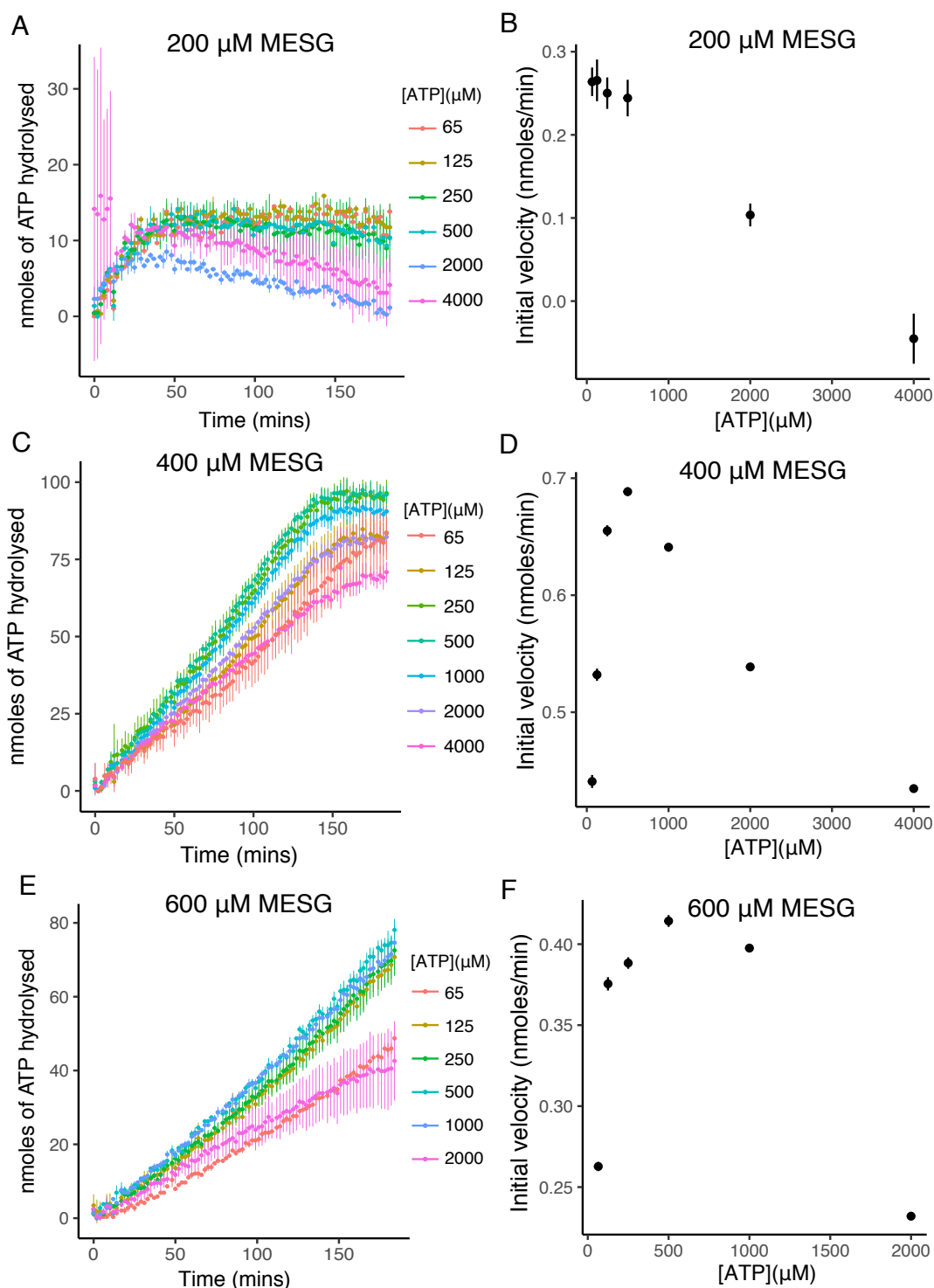
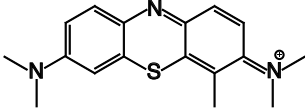
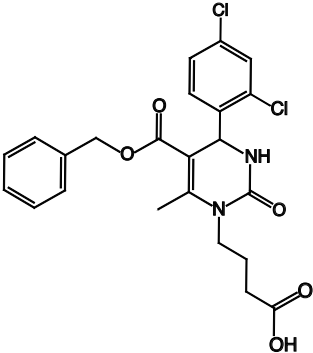
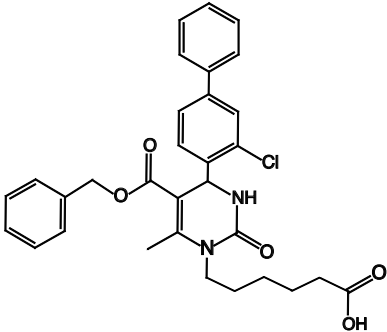
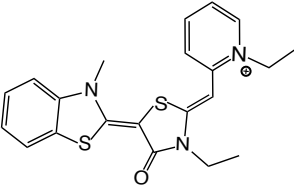
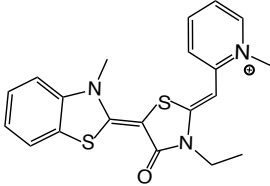
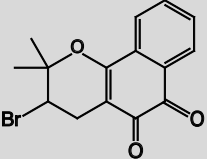


Figure 5.2: The hydrolysis of ATP by PfHsp70-x. A,C,E: Time course of ATP hydrolysis optimising for concentration of MESG and ATP. ATP concentrations are coloured increasingly dark as concentration increases. B,D,F: Initial velocity versus ATP concentration at indicated MESG concentrations.

5.2 Testing Hsp70 inhibitors for PfHsp70-x inhibition

Hsp70 proteins play a role in multiple diseases including cancer [138], neurodegenerative disorders [139] and infectious diseases [170]. Increasing research into therapeutically targeting Hsp70 has resulted in the development of a number of small molecule inhibitors [136, 171, 135]. Here, we test 5 commercially available Hsp70 inhibitors for PfHsp70-x inhibitory activity (Table 5.1). Methylene blue, a phenothiazine dye, has been shown to inhibit heat-induced human Hsp70's through oxidation of an exposed cysteine in the ATPase domain [172]. The dye has also been historically used as an antimalarial and a number of clinical trials have recently been undertaken which find methylene blue to be safe and effective against *Plasmodium falciparum* infections [173, 174, 175]. 115-7c, a model dihydropyrimidine, was found to stimulate the activity of *E. coli* Hsp70, DnaK, and to work in concert with *E. coli* Hsp40, DnaJ, rather than competitively [176]. 116-9e, a bulkier derivative of 115-7c, was found to selectively inhibit activity of the DnaK-DnaJ complex suggesting it was able to interfere with complex formation or prevent the allosteric changes needed for stimulation. MKT-077, a rhodacyanine dye, and its derivative YM-01, exhibit antitumor activity through inhibition of Hsp70s by binding to a pocket near the catalytic site [177, 178, 179]. Additionally, a previously reported specific inhibitor of PfHsp70-x that also has antimalarial activity, BBL, was studied as its mechanism of inhibition is poorly understood [131, 132].

Table 5.1: Hsp70 inhibitors

Inhibitor	Description of mechanism	Structure
Methylene Blue	Irreversibly oxidizes Cys306 in human Hsp72 ATPase domain	
115-7c	Stimulates DnaK ATPase activity	
116-9e	Inhibits stimulation of DnaK ATPase activity	
MKT-077	Allosteric effector of ADP-bound ATPase Binds in pocket near ATP binding pocket	
YM-01	Smaller derivative of MKT-077	
BBL	Specific inhibitor of PfHsp70-x ATPase activity	

The aforementioned MESG assay was used to determine ATP hydrolysis activity of PfHsp70-x_{FL} over a range of inhibitor concentrations (Fig. 5.3). As the dose-response curves did not follow a standard, sigmoidal curve, data could not be fit to a model and thus IC₅₀ values could not be extracted. This lack of sigmoidal shape may be due to a number of binding sites existing or a polyphasic mode of inhibition, which is perhaps to be expected from a highly dynamic, conformation-changing protein. However, we can qualitatively determine the degree of inhibition by described inhibitor concentrations at which ATP hydrolysis activity is reduced to 50%. We can see that methylene blue, BBL, MKT-077 and YM-01 all substantially inhibit PfHsp70-x's ATPase activity with ATP hydrolysis activity reduced to 50% at inhibitor values below 100 μ M. 115-7c, interestingly, shows inhibitory activity, albeit much weaker with ATPase activity reduced to 50% at $\sim$ 250 μ M, despite being a DnaK stimulator. 116-9e shows no inhibition of PfHsp70-x_{FL} even at the highest concentrations. This is consistent with previous studies in which 116-9e did not inhibit DnaK alone but did inhibit DnaK stimulation by DnaJ.

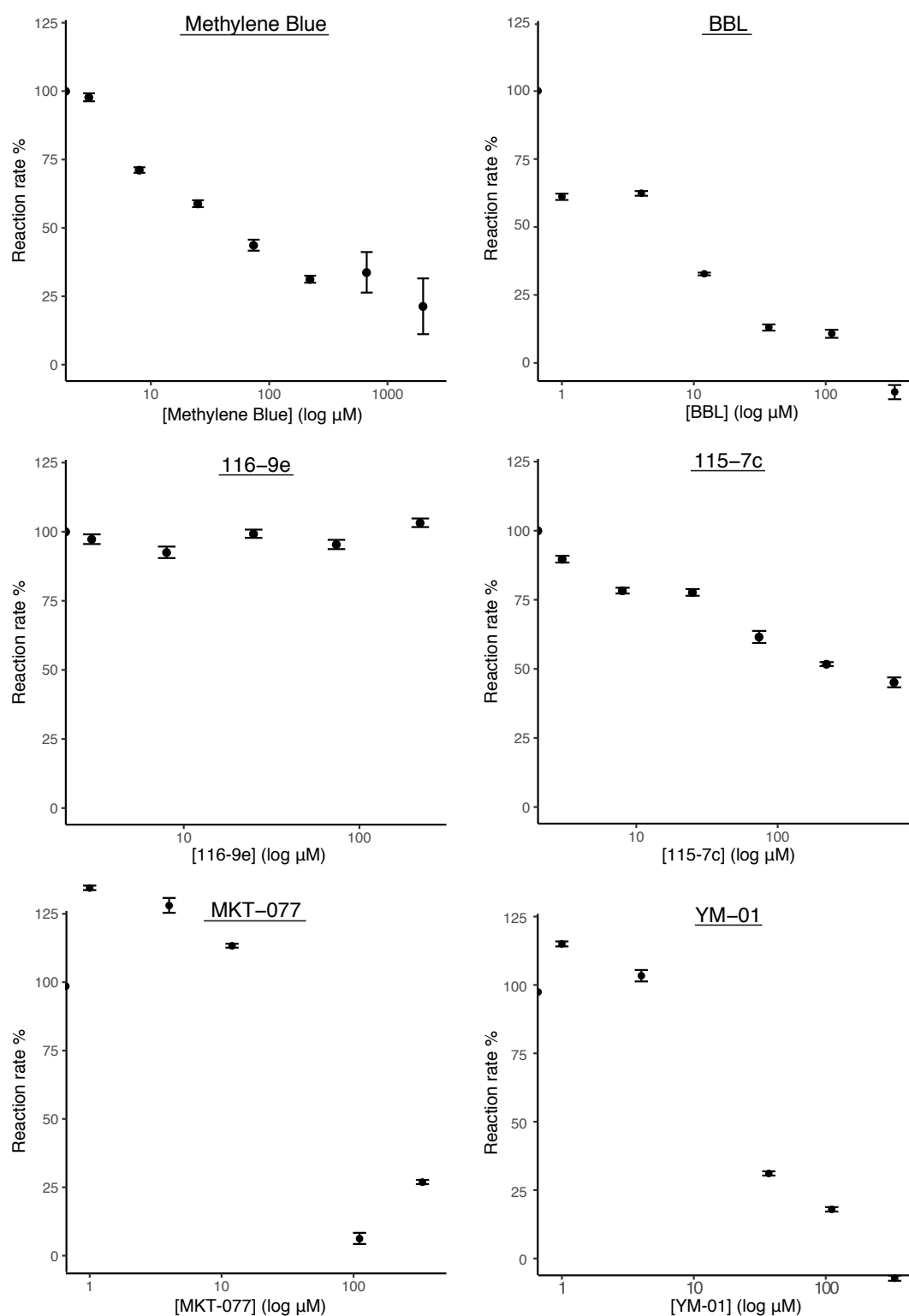


Figure 5.3: Inhibition of PfHsp70-x ATP hydrolysis activity by small molecules. Initial reaction rates shown as a percentage of initial reaction rate with no compound plotted against compound concentration. The mean of three replicates is plotted with error bars indicating standard deviation of replicates.

Hsp70s use cochaperones to stimulate ATPase activity and provide specificity of client proteins [47, 49]. These cochaperones, Hsp40s, are therefore important in tuning the functions of Hsp70 towards pathological roles such as those seen in diseases. Thus, the Hsp70-Hsp40 complex is an attractive target for therapeutics [86]. *Plasmodium falciparum* exports multiple Hsp40s into the host cell, including PFE0055c and PFA0660w, which colocalise with PfHsp70-x in J dots [71]. Previous work has shown PFA0660w to specifically stimulate the activity of PfHsp70-x [91]. To explore the effectiveness of inhibition against stimulated PfHsp70-x, we tested the aforementioned compounds against PfHsp70-x-PFA0660w and PfHsp70-x-PFE0055c (Fig. 5.4–5.6). As the J domain of Hsp40 has previously been shown to be sufficient for Hsp70 ATPase activity stimulation [52], we used PFA0660w and PFE0055c J domains only in our assays.

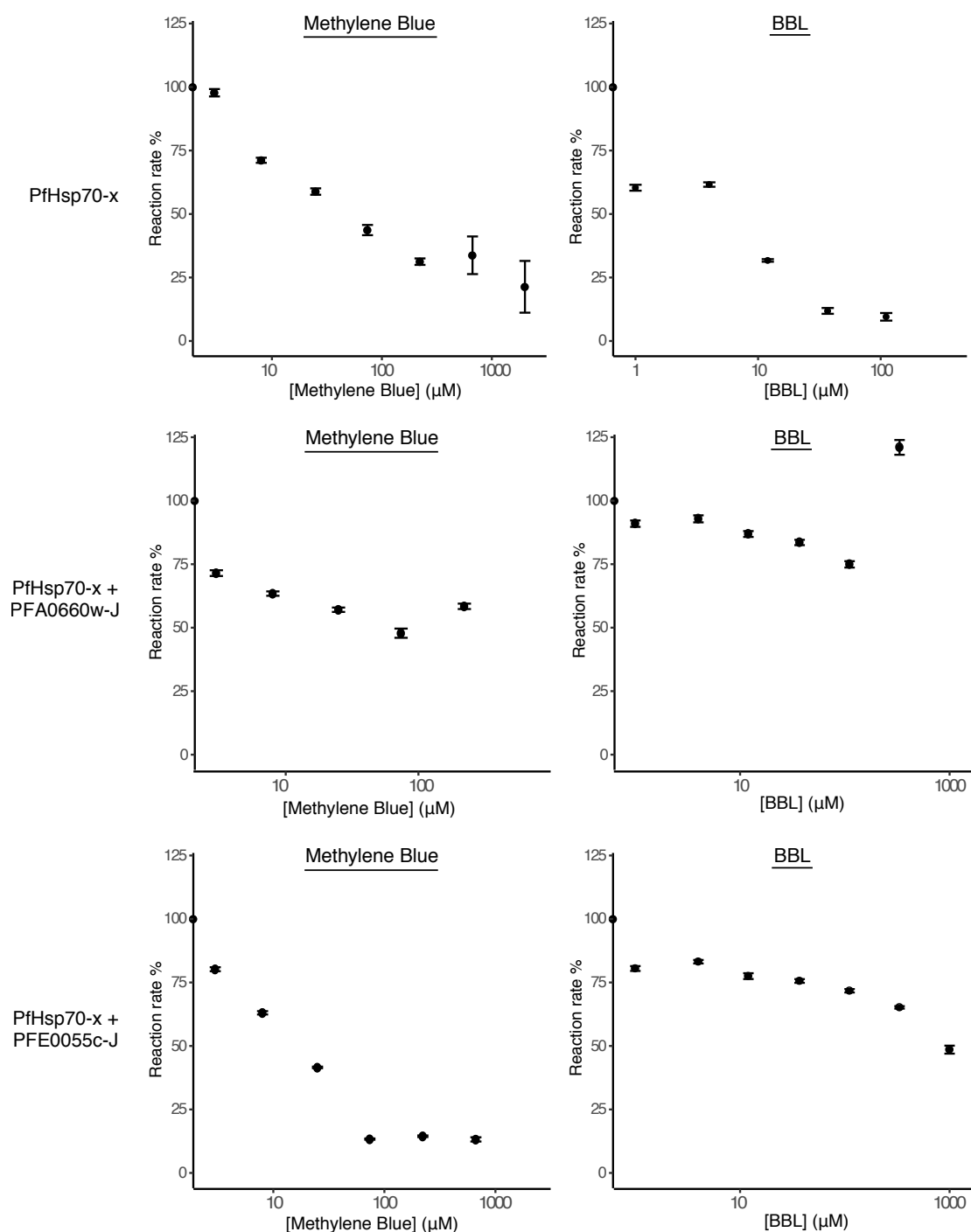


Figure 5.4: Comparison of PfHsp70-x inhibition by methylene blue and BBL in the presence of cochaperones. Top row: methylene blue and BBL inhibition of PfHsp70-x alone. Middle row: inhibition of PfHsp70-x in the presence of PFA0660w_J. Bottom row: inhibition of PfHsp70-x in the presence of PFE0055c_J. Reaction rate % calculated as a percentage of initial reaction rate without compound (100%). The mean of three replicates is plotted with error bars indicating standard deviation of replicates.

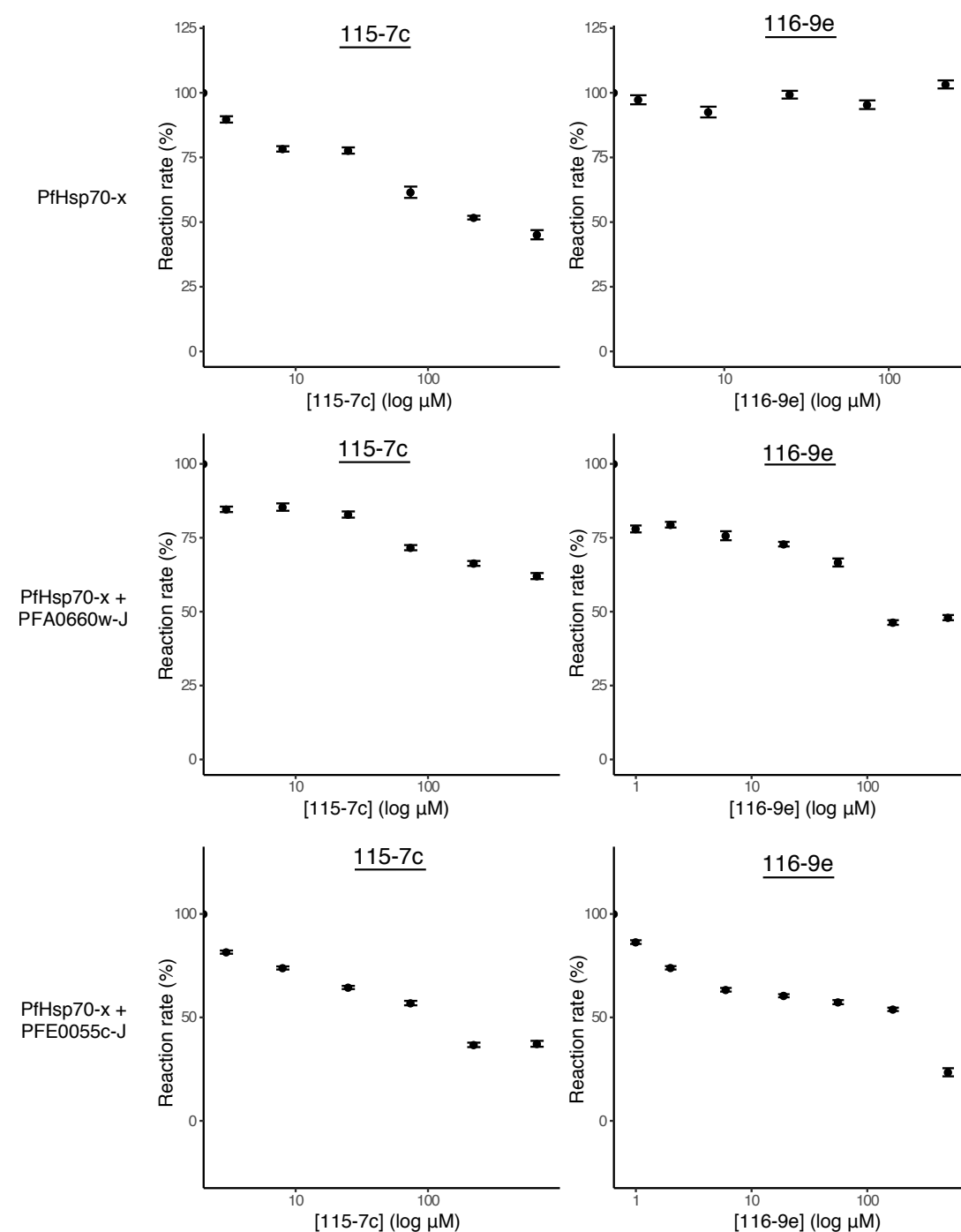


Figure 5.5: Comparison of PfHsp70-x inhibition by dihydropyrimidines in the presence of cochaperones. Top row: 115-7c and 116-9e inhibition of PfHsp70-x alone. Middle row: inhibition of PfHsp70-x in the presence of PFA0660w-J. Bottom row: inhibition of PfHsp70-x in the presence of PFE0055c-J. Reaction rate % calculated as a percentage of initial reaction rate without compound (100%). The mean of three replicates is plotted with error bars indicating standard deviation of replicates.

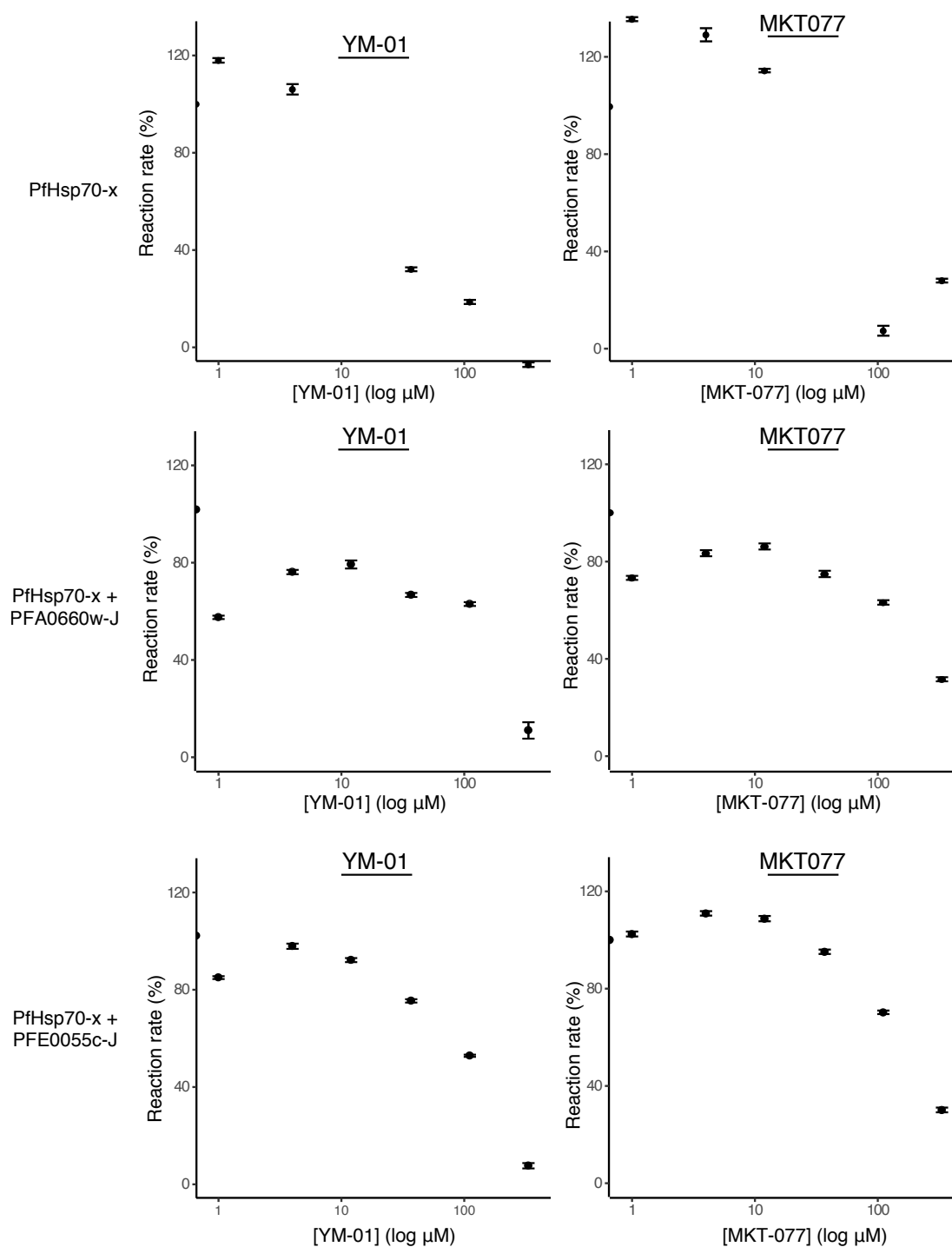


Figure 5.6: Comparison of PfHsp70-x inhibition by MKT-077 and YM-01 in the presence of cochaperones. Top row: YM-01 and MKT-077 inhibition of PfHsp70-x alone. Middle row: inhibition of PfHsp70-x in the presence of PFA0660w_J. Bottom row: inhibition of PfHsp70-x in the presence of PFE0055c_J. Reaction rate % calculated as a percentage of initial reaction rate without compound (100%). The mean of three replicates is plotted with error bars indicating standard deviation of replicates.

In Figure 5.4, we compare the inhibitory activities of methylene blue and BBL in the absence and presence of PFA0660w_J and PFE0055c_J. For methylene blue, we see inhibition is virtually absent when PfHsp70-x is stimulated by PFA0660w_J and yet still inhibits PFE0055c_J-stimulated activity. Interestingly, BBL also shows differential inhibition dependent on the presence of a J domain. For both PFA0660w- and PFE0055c-stimulated activity, we see reduced inhibition, with significant inhibition (>50% activity) occurring at <1000 μ M as opposed to <10 μ M for unstimulated PfHsp70-x.

Next, we look to the dihydropyrimidines: 115-7c and 116-9e (Fig. 5.5). As previously discussed, 115-7c inhibited PfHsp70-x activity, albeit weakly, despite it being a DnaK stimulator. This level of inhibition remains consistent in the presence of PFE0055c_J with substantial inhibition (>50%) at <500 μ M. However, we see reduced inhibition in the presence of PFA0660w_J; reaction rates do not fall below 50% of the compound-free rate even at the highest concentrations of 115-7c. 116-9e showed no inhibition of PfHsp70-x alone but does inhibit stimulated PfHsp70-x activity. For both PFA0660w_J and PFE0055c_J-stimulated activity, 116-9e significantly inhibits ATP hydrolysis at < 100 μ M and < 500 μ M, respectively. It seems that 116-9e behaves in a similar fashion for various Hsp70-Hsp40 complexes, whereas 115-7c can inhibit or stimulate activity depending on the Hsp70.

The rhodacyanine dyes, YM-01 and MKT-077, strongly inhibit PfHsp70-x ATPase activity. This inhibition is weakened upon addition of both PFA0660w and PFE0055c J domains with substantial inhibition occurring at < 500 μ M compared < 100 μ M for unstimulated PfHsp70-x activity (Fig. 5.6).

5.3 The mechanism of inhibition

Methylene blue is believed to selectively inhibit heat-induced Hsp70s by oxidation of a cysteine not present in constitutively expressed Hsp70s [172]. A multiple sequence alignment performed of PfHsp70-x, Hsc70 (human: constitutively expressed) and Hsp72 (human: heat inducible) reveals that PfHsp70-x has a cysteine (C337) that, according to Miyata *et al*, indicates PfHsp70-x has the ability to act as a redox sensor

(Fig. 5.7). The multiple sequence alignment also reveals a number of additional cysteines not present in either of the human hsp70s (C50, C271, C391). Locating these residues on the crystal structure of PfHsp70-x_{ATPase}, we can observe that C337 is not the most solvent accessible cysteine but that C391 with a solvent accessible surface area of 34.31 Å², followed by C50 with 12.75 Å² (Table 5.2). Following the logic of Miyata that the presence of accessible cysteines suggests the ability of oxidative stress sensing, we may therefore postulate that PfHsp70-x has an additional chemical moiety to sense oxidative stress. The additional solvent exposed cysteine may also result in a different mechanism of methylene blue inhibition than that proposed for Hsp72. Intrigued if this was the case, we soaked crystals of PfHsp70-x_{ATPase}:AMP-PNP in high concentrations of methylene blue (1 - 10 mM) for 16 hours and collected diffraction data. Several data sets from a number of soaked crystals were collected and analysed for changes in structure, the presence of electron density corresponding to methylene blue and for cysteine modifications. However, no changes could be seen in any of the data sets. All cysteines, both non-conserved and conserved, were examined closely for evidence of additional electron density caused by oxidation of the sulfhydryl group but the electron density fit the cysteine side chains well with no positive density seen in the Fo-Fc difference electron density maps (Fig. 5.8). Perhaps methylene blue is not inhibiting PfHsp70-x by the same mechanism as that proposed for human Hsp72 or, alternatively, the compound was not able to fully enter the solvent channels and access reaction sites.

Table 5.2: Solvent accessibility of PfHsp70-x_{ATPase} cysteines.

Residue	Residue solvent accessible surface area (Å²)
C50	12.75
C271	0.00
C337	0.40
C391	34.31

<i>PfHsp70-x</i>	30	EESEVAIGIDLGT	TS	SCVGI	C	R	N	G	V	V	D	I	I	A	N	D	Q	G	N	R	T	T	P	S	Y	V	A	F	T	D	T	E	R	L	I	G	D	A	A	K	N	Q	A	S	R	N	P	E	N	T	95																	
<i>Hsc70</i>	1	MSKGP	AVG	I	D	L	G	T	T	Y	SCVGV	F	Q	H	G	K	V	E	I	I	A	N	D	Q	G	N	R	T	T	P	S	Y	V	A	F	T	D	T	E	R	L	I	G	D	A	A	K	N	Q	V	A	M	N	P	T	N	T	66										
<i>Hsp72</i>	1	MAKAA	A	I	G	I	D	L	G	T	T	Y	SCVGV	F	Q	H	G	K	V	E	I	I	A	N	D	Q	G	N	R	T	T	P	S	Y	V	A	F	T	D	T	E	R	L	I	G	D	A	A	K	N	Q	V	A	L	N	P	Q	N	T	66								
<i>PfHsp70-x</i>	96	V	F	D	A	K	R	L	I	G	R	K	F	S	E	T	T	V	Q	S	D	M	K	H	W	P	F	T	V	K	G	G	S	D	G	K	P	M	I	E	V	S	Y	Q	G	E	K	T	F	H	P	E	E	I	S	S	M	V	L	K	M	K	E	V	A	161		
<i>Hsc70</i>	67	V	F	D	A	K	R	L	I	G	R	R	F	D	D	A	V	V	Q	S	D	M	K	H	W	P	F	M	V	V	N	D	-	A	G	R	P	K	V	Q	V	E	Y	K	G	E	T	K	S	F	Y	P	E	E	V	S	S	M	V	L	T	K	M	K	E	I	A	131
<i>Hsp72</i>	67	V	F	D	A	K	R	L	I	G	R	K	F	G	D	P	V	V	Q	S	D	M	K	H	W	P	F	Q	V	I	N	D	-	G	D	K	P	K	V	Q	V	S	Y	K	G	E	T	K	A	F	Y	P	E	E	I	S	S	M	V	L	T	K	M	K	E	I	A	131
<i>PfHsp70-x</i>	162	E	T	Y	L	G	K	P	V	K	N	A	V	I	T	V	P	A	Y	F	N	D	S	Q	R	Q	A	T	K	D	A	G	A	I	A	G	L	N	V	L	R	I	I	N	E	P	T	A	A	A	I	A	Y	G	L	D	K	K	G	K	G	E	Q	N	I	L	227	
<i>Hsc70</i>	132	E	A	Y	L	G	K	T	V	T	N	A	V	T	V	P	A	Y	F	N	D	S	Q	R	Q	A	T	K	D	A	G	T	I	A	G	L	N	V	L	R	I	I	N	E	P	T	A	A	A	I	A	Y	G	L	D	K	K	V	G	A	E	R	N	V	L	I	197	
<i>Hsp72</i>	132	E	A	Y	L	G	P	V	T	N	A	V	I	T	V	P	A	Y	F	N	D	S	Q	R	Q	A	T	K	D	A	G	V	I	A	G	L	N	V	L	R	I	I	N	E	P	T	A	A	A	I	A	Y	G	L	D	R	T	G	K	G	E	R	N	V	L	I	197	
<i>PfHsp70-x</i>	228	F	D	L	G	G	G	T	F	D	V	S	I	L	T	I	E	D	G	I	F	E	V	K	A	T	S	G	D	T	H	L	G	G	E	D	F	D	N	K	L	V	N	F	C	V	Q	D	F	K	K	K	N	G	G	K	D	V	S	K	N	S	K	S	L	R	R	293
<i>Hsc70</i>	198	F	D	L	G	G	G	T	F	D	V	S	I	L	T	I	E	D	G	I	F	E	V	K	S	T	A	G	D	T	H	L	G	G	E	D	F	D	N	R	M	V	N	H	F	I	A	E	F	K	R	K	H	-	K	K	D	I	S	E	N	K	R	A	V	R	R	262
<i>Hsp72</i>	198	F	D	L	G	G	G	T	F	D	V	S	I	L	T	I	D	D	G	I	F	E	V	K	A	T	A	G	D	T	H	L	G	G	E	D	F	D	N	R	L	V	N	H	F	V	E	E	F	K	R	K	H	-	K	K	D	I	S	Q	N	K	R	A	V	R	R	262
<i>PfHsp70-x</i>	294	L	R	T	Q	C	E	K	A	K	R	V	L	S	S	S	A	Q	A	T	I	E	V	D	S	L	F	D	G	I	D	Y	N	V	N	I	T	R	A	K	F	E	E	L	C	M	D	Q	F	R	N	T	L	I	P	V	E	K	V	L	K	D	A	K	M	D	K	359
<i>Hsc70</i>	263	L	R	T	A	C	E	R	A	K	R	T	L	S	S	S	T	Q	A	S	I	E	I	D	S	L	Y	E	G	I	D	F	Y	T	S	I	T	R	A	R	F	E	E	L	N	A	D	L	F	R	G	T	L	D	P	V	E	K	A	L	R	D	A	K	L	D	K	328
<i>Hsp72</i>	263	L	R	T	A	C	E	R	A	K	R	T	L	S	S	S	T	Q	A	S	L	E	I	D	S	L	F	E	G	I	D	F	Y	T	S	I	T	R	A	R	F	E	E	L	C	S	D	L	F	R	S	T	L	E	P	V	E	K	A	L	R	D	A	K	L	D	K	328
<i>PfHsp70-x</i>	360	S	Q	V	H	E	I	V	L	V	G	G	S	T	R	I	P	K	I	Q	Q	L	I	K	D	F	F	N	G	K	E	P	C	K	A	I	N	P	D	E	A	V	A	Y	G	A	A	V	Q	A	A	I	L	S	G	D	Q	S	S	A	V	K	D	L	L	L	L	425
<i>Hsc70</i>	329	S	Q	I	H	D	I	V	L	V	G	G	S	T	R	I	P	K	I	Q	K	L	L	Q	D	F	F	N	G	K	E	L	N	K	S	I	N	P	D	E	A	V	A	Y	G	A	A	V	Q	A	A	I	L	S	G	D	K	S	E	N	V	Q	D	L	L	L	L	394
<i>Hsp72</i>	329	A	Q	I	H	D	L	V	L	V	G	G	S	T	R	I	P	K	V	Q	K	L	L	Q	D	F	F	N	G	R	D	L	N	K	S	I	N	P	D	E	A	V	A	Y	G	A	A	V	Q	A	A	I	L	M	G	D	K	S	E	N	V	Q	D	L	L	L	L	394

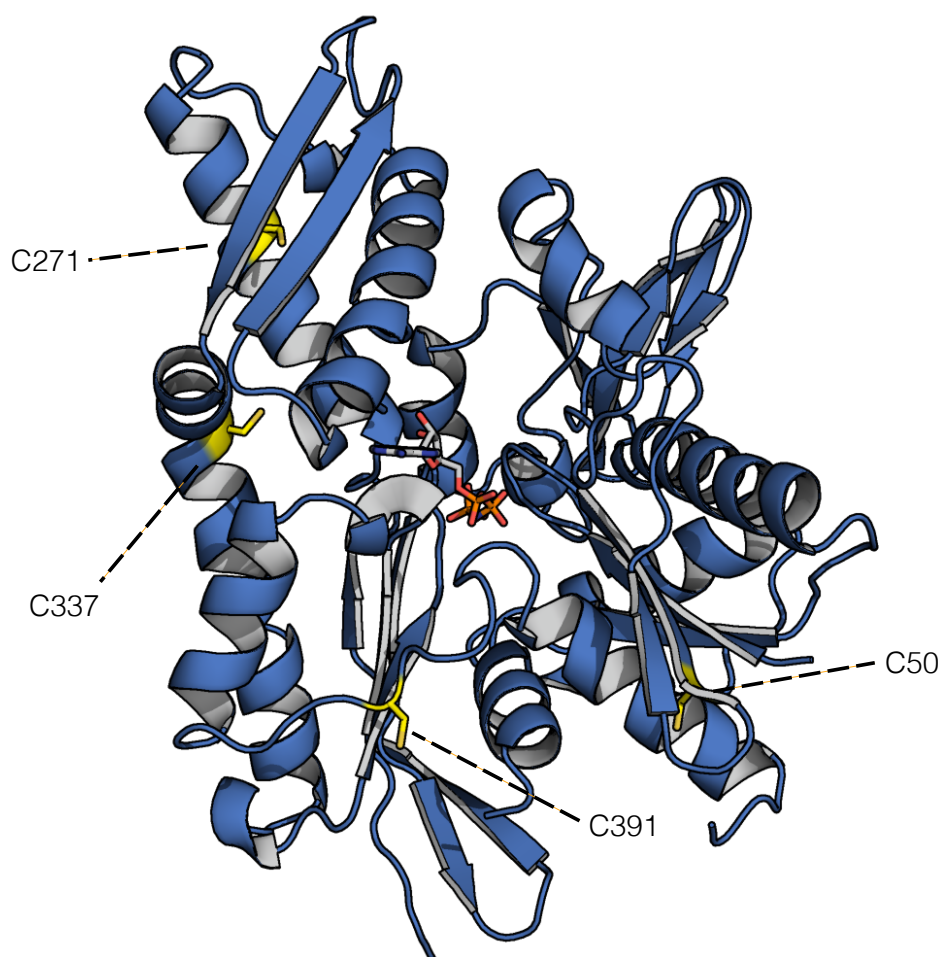


Figure 5.7: Cysteines of PfHsp70-x_{ATPase}. Top: Multiple sequence alignment by MAFFT [180, 181] of PfHsp70-x, Hsc70 and Hsp72 amino acid sequences coloured by conservation. The redox-sensing cysteine of heat-inducible Hsp72 is indicated in green and PfHsp70-x specific cysteines highlighted in yellow. Bottom: Crystal structure of PfHsp70-x_{ATPase} with cysteines highlighted in yellow with side chains represented as sticks.

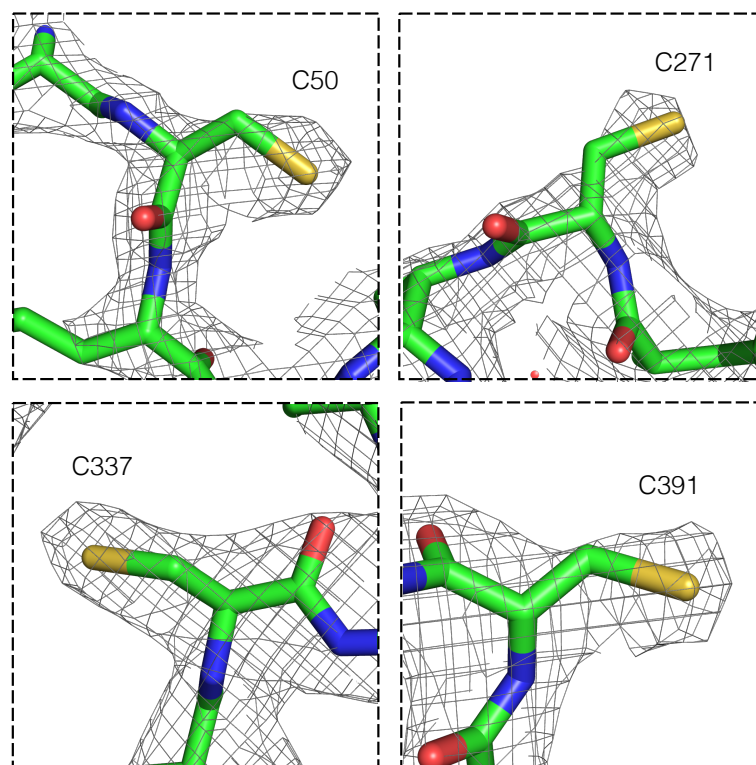


Figure 5.8: Lack of cysteine modification upon methylene blue treatment. Representative examples of cysteines in PfHsp70-x_{ATPase} and associated electron density (2FOFCWT PH2FOFCWT, sigma level 2 RMSD). Protein is shown in stick representation and coloured according to element: nitrogen coloured blue; oxygen coloured red; sulphur coloured yellow; carbon coloured green.

5.4 Studying ligand binding with STD-NMR

The mechanism of BBL inhibition of PfHsp70-x is unknown and attempts to cocrystallise or soak PfHsp70-x_{ATPase} with BBL were unsuccessful. Methylene blue inhibition of PfHsp70-x also remains unclear as soaking PfHsp70-x_{ATPase} crystals with methylene blue indicated cysteines were not oxidised as is the case for Hsp72. Therefore, we aimed to gain insights into the mechanism of inhibition by both methylene blue and BBL through confirming which domain the compounds were binding.

To assess the inhibitors methylene blue and BBL are binding to the ATPase domain of PfHsp70-x as predicted, we used saturation transfer difference (STD) NMR. STD-NMR is a useful technique used to characterise weak ligand binding

(K_D mM – μ M) to proteins in the absence of any labels or tags and requires low concentrations of protein ($<10 \mu$ M)[108]. In the context of a weak-binding ligand, there is exchange between protein-bound and unbound states with the equilibrium of these states dependent on the amount of ligand present. STD-NMR relies on the nuclear Overhauser effect in which spin polarization is transferred from one nucleus to another through space, not through chemical bonds. To transfer spin polarization from protein to ligand, the protein must be selectively saturated by irradiating at a region of the 1D ^1H spectrum containing protein-only resonances; the resultant spectrum is referred to as the ‘on-resonance’ spectrum. Then, to calculate the saturation transfer difference, we must also collect an ‘off-resonance’ spectrum in which the protein is not saturated because irradiation occurs where there are no resonances (> 40 ppm). The on-resonance spectrum is then subtracted from the off-resonance spectrum resulting in a difference spectrum containing only signals of ligand resonances that received saturation transfer from the protein (Fig. 5.9).

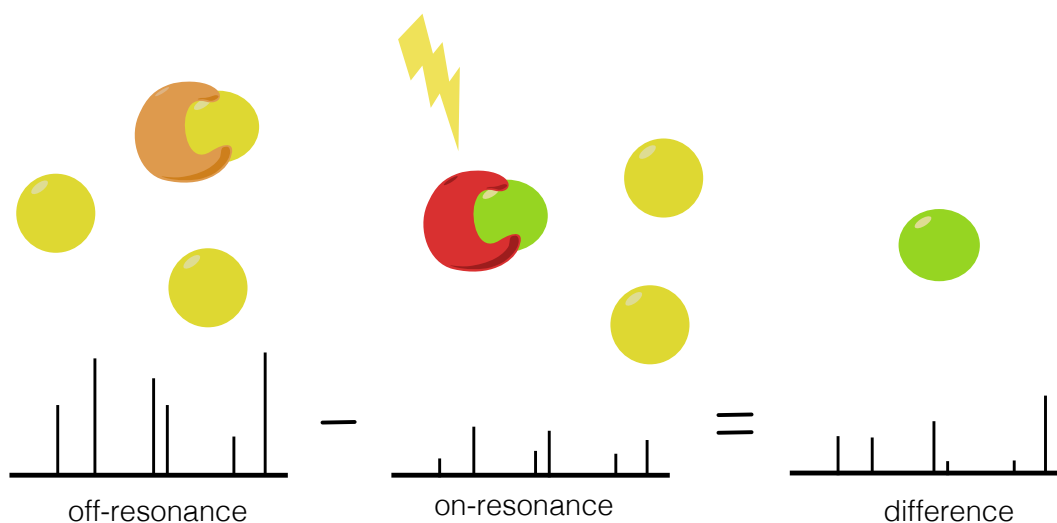


Figure 5.9: Schematic of saturation transfer difference NMR. Diagram illustrating ligand binding (yellow) to receptor protein (orange). An example off-resonance 1D ^1H spectrum of the ligand is shown below. An on-resonance spectrum is shown illustrating irradiation of protein (red) leading to magnetization transfer to ligand (green) leading to reduction in peak intensity as indicated by black triangles. The on-resonance spectrum is subtracted from the off-resonance spectrum resulting in a difference spectrum.

To determine if methylene blue and BBL were binding to the ATPase domain or elsewhere on PfHsp70-x, we performed STD-NMR experiments with PfHsp70-x_{FL}

and PfHsp70-x_{ATPase}. Ligand concentrations were varied to calculate K_D values. Comparing 1D ^1H NMR spectra of methylene blue and BBL to predicted spectra calculated from their chemical structures, we can assign resonances to protons in the chemical structure (Fig. 5.10 and Fig. 5.11). The indicated resonances were integrated in off-resonance (reference) and difference spectra to allow calculation of the STD amplification factor. STD amplification factor was then plotted against ligand concentration for each resonance to allow estimation of K_D by fitting a Michaelis-Menten-like model with the data (Fig. 5.12) and Fig. 5.13).

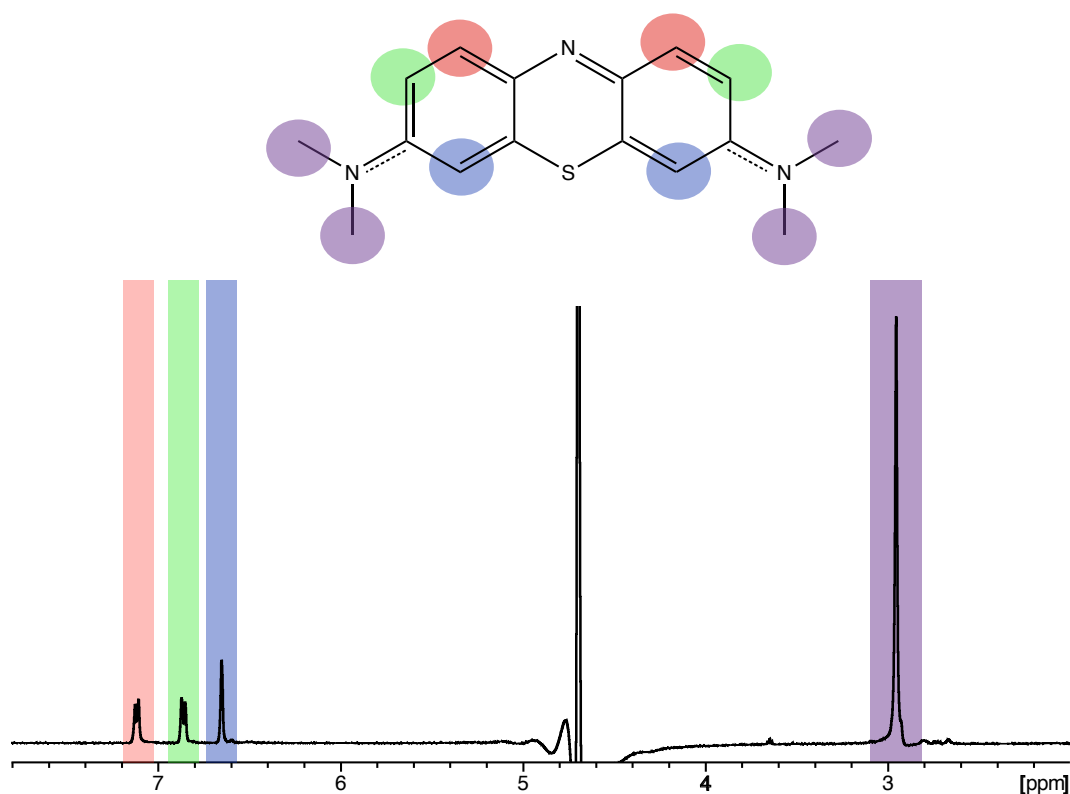


Figure 5.10: 1D ^1H spectrum of methylene blue. Recorded 1D ^1H spectrum of methylene blue with resonances used in STD calculations indicated by coloured boxes. Protons corresponding to resonances are indicated on the chemical structure of methylene blue and coloured accordingly.

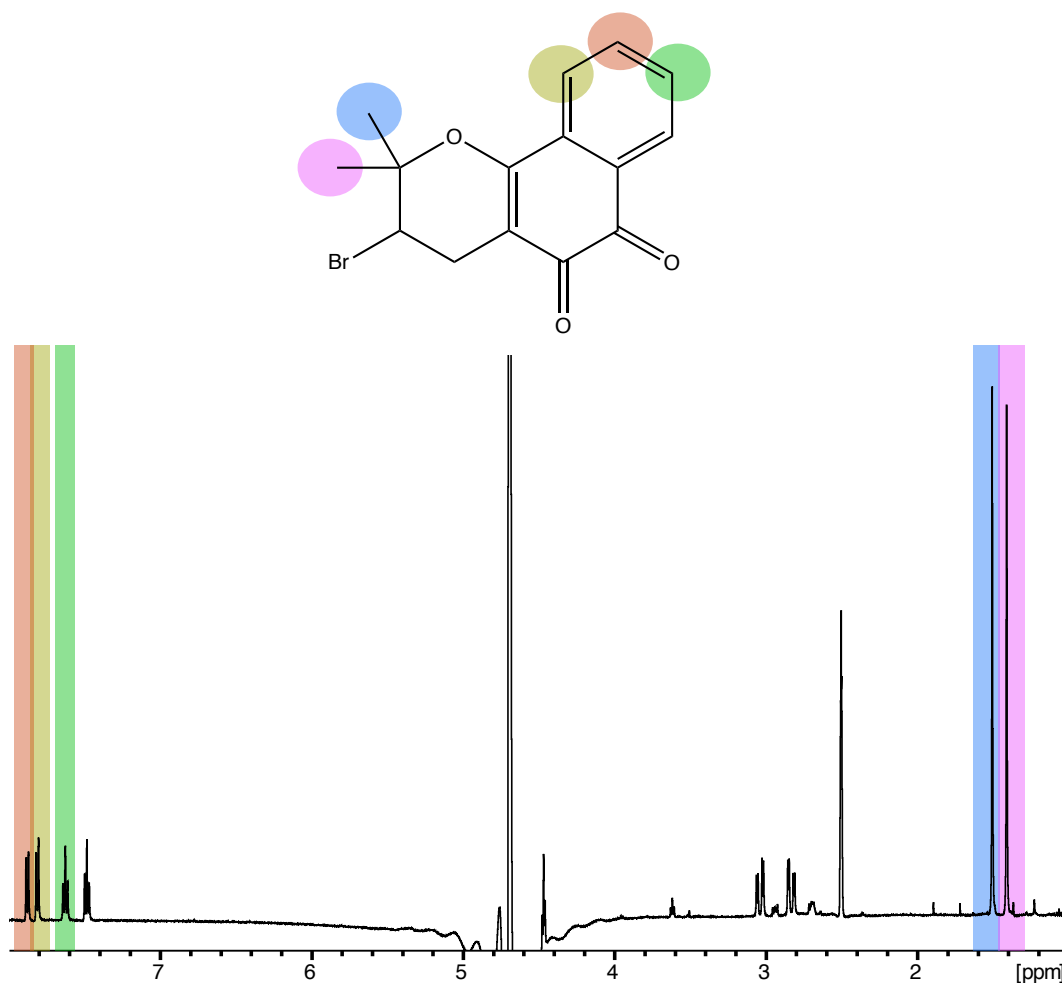


Figure 5.11: 1D ¹H spectrum of BBL. Recorded 1D ¹H spectrum of BBL with resonances used in STD calculations indicated by coloured boxes. Protons corresponding to resonances are indicated on the chemical structure of BBL and coloured accordingly.

We can see that methylene blue binds to both PfHsp70-x_{FL} and PfHsp70-x_{ATPase} with very similar affinities: 271 μ M and 151 μ M (Fig. 5.12). This suggests to us that methylene blue does bind to the ATPase domain as described in previous studies. We see higher STD_{amp} values for PfHsp70-x_{FL} as this protein is larger than the ATPase domain alone and thus can more efficiently transfer magnetisation.

BBL also bound to PfHsp70-x_{ATPase} as well as to PfHsp70-x_{FL} with K_D values within the error of each other. There is a larger degree of error for the estimation of BBL's K_D upon binding to PfHsp70-x as binding sites were not saturated and therefore it was harder to fit the Michaelis-Menten-like model.

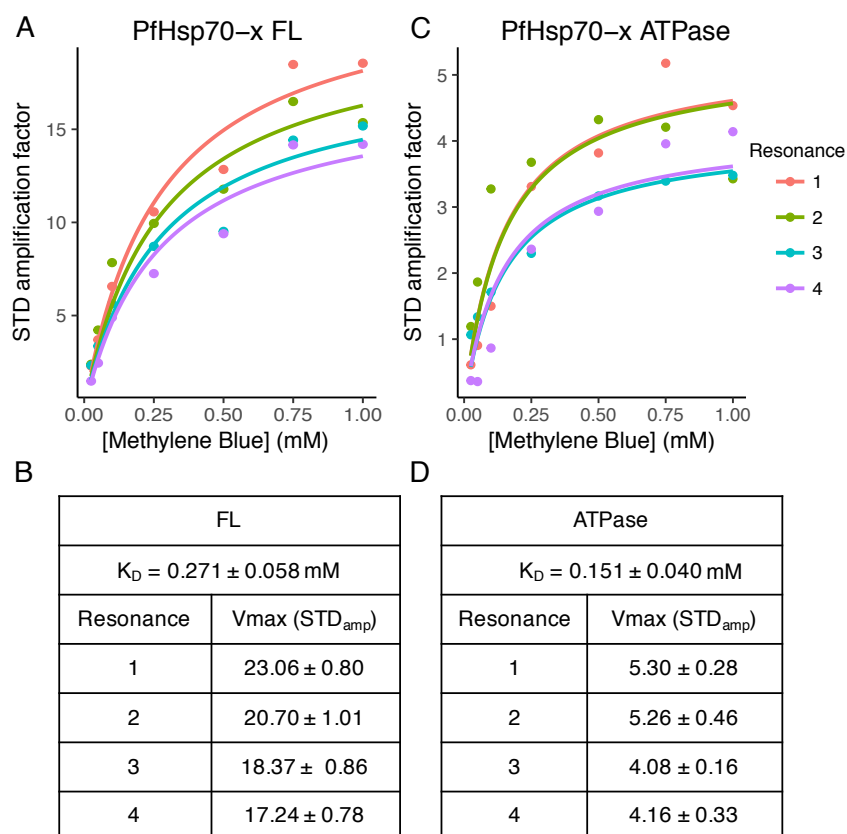


Figure 5.12: Calculating K_D s of methylene blue with PfHsp70-x_{FL} and PfHsp70-x_{ATPase}. **A:** STD amplification factor plotted against methylene blue concentration for PfHsp70-x_{FL} shown for each resonance. Michaelis-Menten-like fits are shown for each resonance. **B:** Estimated K_D for methylene blue binding to PfHsp70-x_{FL} and STD_{amp max} values for each resonance. **C:** STD amplification factor plotted against methylene blue concentration for PfHsp70-x_{ATPase} shown for each resonance. Michaelis-Menton fits are shown for each resonance. **D:** Estimated K_D for methylene blue binding to PfHsp70-x_{ATPase} and STD_{amp} values for each resonance.

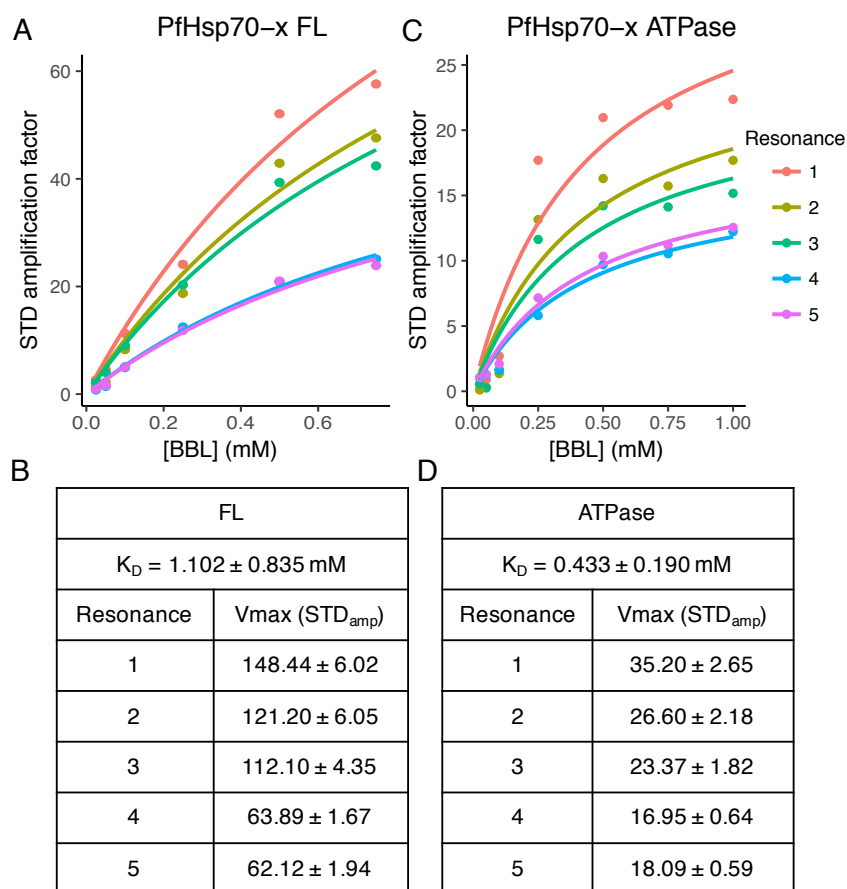


Figure 5.13: Calculating K_D s of BBL with PfHsp70-x_{FL} and PfHsp70-x_{ATPase}. **A:** STD amplification factor plotted against BBL concentration for PfHsp70-x_{FL} shown for each resonance. Michaelis-Menten-like fits are shown for each resonance. **B:** Estimated K_D for BBL binding to PfHsp70-x_{FL} and STD_{amp} values for each resonance. **C:** STD amplification factor plotted against BBL concentration for PfHsp70-x_{ATPase} shown for each resonance. Michaelis-Menten fits are shown for each resonance. **D:** Estimated K_D for BBL binding to PfHsp70-x_{ATPase} and STD_{amp} values for each resonance.

5.5 Discussion

In this chapter, we have demonstrated that a number of commercially available Hsp70s inhibitors are also capable of inhibiting the ATPase activity of PfHsp70-x (Fig. 5.14). Particularly interesting amongst these are methylene blue and the dihydropyrimidines: 115-7c and 116-9e. Additionally, we aimed to further our understanding of BBL inhibition; a specific inhibitor of PfHsp70-x.

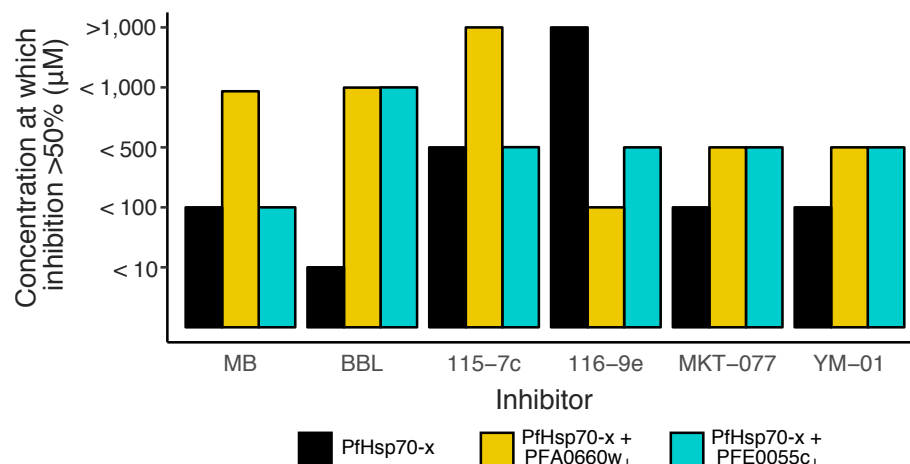


Figure 5.14: Summary of stimulated and unstimulated PfHsp70-x inhibition.

A qualitative summary of inhibitory compounds of PfHsp70-x comparing compounds methylene blue (MB), BBL, 115-7c, 116-9e, MKT-077 and YM-01. Inhibition is shown for unstimulated PfHsp70-x and stimulated PfHsp70-x (by PFA0660wJ and PFE0055cJ).

Methylene blue, a commonly used dye, has received renewed attention as an effective and affordable antimalarial [174]. When methylene blue is used in artemisinin-based combination therapy (ACT) alongside artesunate, treatment efficacy is significantly superior to that of the standard ACT regimen in sub-Saharan Africa [175]. The methylene blue combination therapies could provide a cheaper alternative for treatment of *Plasmodium falciparum* infection and be particularly effective against drug-resistant strains as methylene blue is a broadly acting antimalarial. Methylene blue is known to inhibit *P. falciparum* glutathione reductase [182] and heme biocrystallization [183] with other possible mechanisms, which makes it a difficult drug to develop resistance against. Here we provide an additional putative mechanism of action through the inhibition of parasitic Hsp70s.

We observed that PfHsp70-x's ATPase activity is substantially inhibited by methylene blue with activity being inhibited to >50% at <100 μ M of the dye. Interestingly, this inhibition was absent upon stimulation by PFA0660w but not PFE0055c. Previously published studies examining the mechanism of Hsp70 inhibition by methylene blue suggested that methylene blue oxidises a solvent accessible cysteine only found in the ATPase domain of heat-inducible Hsp70s [172]. This cysteine was found to be present in PfHsp70-x by multiple sequence

alignment with human Hsp72 and Hsc70 (heat-induced and constitutively expressed, respectively) alongside additional cysteines with substantial solvent accessibility. However, soaking PfHsp70-x_{ATPase} crystals with methylene blue did not cause modification of any cysteines on the ATPase domain of PfHsp70-x. This finding suggested to us that methylene blue may inhibit through another mechanism, perhaps by modifying the substrate binding domain. To test our theory, STD-NMR was employed to estimate the K_D of methylene blue binding to PfHsp70-x_{ATPase} and PfHsp70-x_{FL}. If methylene blue did in fact work by binding to the substrate binding domain of PfHsp70-x, then we would see a lower K_D , and therefore a higher affinity, for the full-length protein. Interestingly, we saw that methylene blue binds to the ATPase domain of PfHsp70-x as well as it does to the full-length protein indicating that it inhibits PfHsp70-x activity by binding to only the ATPase domain. Perhaps this was not observed by X-ray crystallography as methylene blue binding might require the ATPase domain to be able to ‘breathe’ and allow exposure of certain binding sites as would happen in solution. However, in a crystal, a protein’s structure is extremely restrained and any movements are minimal which may prevent compounds such as methylene blue binding and being able to chemically modify PfHsp70-x. This highlights the importance of using solution-based techniques alongside X-ray crystallography in the discovery and understanding of ligand binding.

We also aimed to gain insights into the mechanism of BBL; a supposedly ‘specific’ and strong inhibitor of PfHsp70-x. BBL inhibited the ATPase activity of PfHsp70-x to < 50% of normal activity at a concentration below 10 μ M. Using STD-NMR we confirmed that BBL binds to the ATPase domain of PfHsp70-x albeit relatively weakly with a K_D in the order of 1 mM. We also observed that in the presence of J domains from cochaperones PFA0660w and PFE0055c, BBL’s inhibitory action is greatly reduced implying that the compound binds near the Hsp70-Hsp40 interface where it competes with Hsp40s or that the compound binds less favourably to the cochaperone-bound PfHsp70-x state.

115-7c and 116-9e, small compounds designed to modulate *E. coli* DnaK activity, were also tested in our ATPase assay for activity against PfHsp70-x. 115-7c was originally discovered as a stimulator of DnaK ATPase activity [176] but we surprisingly observed it to act as an inhibitor of PfHsp70-x. 116-9e, however, showed similar inhibition patterns for PfHsp70-x as it did for DnaK; 116-9e is not an inhibitor of basal ATPase activity but only of cochaperone stimulated activity. Through the action of these dihydropyrimidines, we can see how Hsp70s can be specifically and differentially inhibited by small molecules despite their high amino acid conservation and similar structures. We can begin to understand and exploit these differences by comparing non-conserved patches and residues believed to be involved in 115-7c binding. In Wisén *et al* 2010, NMR was used to estimate the binding site of 115-7c on the ATPase domain of DnaK and found the binding site to be adjacent to but distinct from where Hsp40 J domains are predicted to bind. By performing a pairwise alignment of PfHsp70-x and DnaK, we can observe differences in sequence that may explain the different behaviour of 115-7c. Residues implicated in 115-7c binding to DnaK are indicated in Figure 5.15 and we can see that the majority are conserved except for the arginine 188 to glutamine change. We can also see that this amino acid change is within a small region of poor conservation which lays on a loop within the structure and could be accounting for the differences seen. When mapping the residues believed to be involved in 115-7c binding onto the structure of PfHsp70-x it is possible to see the 115-7c binding patch (pink) is in close proximity to poorly conserved areas. 116-9e is believed to bind at or near the same site as 115-7c although their effects on PfHsp70-x activity are different. It is feasible that the binding site of 115-7c overlaps with that of PFA0660w causing competition and thus preventing stimulation of activity. This would then suggest that either 115-7c or PFA0660w, or perhaps even both, bind in a different manner to DnaK stimulated by DnaJ.

PfHsp70-x 32 SEVAIGIDLGTTYS[●]CVGICRNGVVDIIANDQGNRTTPSYVAFITD-TERLIGDAAKNOASRN[●]PENTVF 97
DnaK 1 MGKILGIDLGITNS[●]CVAILMDGTTPRVLENAEGDRTTPSI[●]IAYTQDGETLVGQPAKROAVTNPQNTLF 67

PfHsp70-x 98 DAKRLIGRKFS[●]ETT[●]VSDMKHWPFTVKGGSDGKPMIEVSYQGEKKT[●]FHP[●]EIS[●]SMVLKKMK[●]EEVAET[●]Y 164
DnaK 68 AIKRLIGRRFQDEEVQR[●]VSIMPFKIIAADNGDAWVEVKG----QKMA[●]PQISAEVLKKMK[●]KTAEDY 130

PfHsp70-x 165 LGKPVKNAVITVPAYFND[●]SORQATKDAGAIAGLNVLRIINEPTAAAIAYGLDKK[●]KG[●]EQN[●]ILIFDLG 231
DnaK 131 LGEPVTEAVITVPAYFNDA[●]QROATKDAGR[●]IAGLEV[●]KRIINEPTAAALAYGLDK-GTGNRT[●]IAVYDLG 196

PfHsp70-x 232 GGTFDVSL[●]LTLE---DGIFEVKATSGDTHLGGEDF[●]DNKLVNFCVQDFKK[●]KN[●]GK[●]GVSKNSKSLRR 294
DnaK 197 GGTFD[●]IS[●]IEIDEVDG[●]EKTFEVLATNGDTHLGGEDF[●]SRLINYLVEEFKK[●]DQ[●]GIDLRNDPLAMQRL 262

PfHsp70-x 295 RTQCEKAKRVLSSAQA[●]TI[●]EVDSL---FDGIDYVNI[●]TRAK[●]FEELCMDOFRNT[●]LI[●]PVEKVLKDAKM 357
DnaK 263 KEAAEKAKIELSSAQ[●]QTDVNL[●]PYITADATGPKHMI[●]KVTRAK[●]LSLVEDLVNRSIEPLKVALQDAGL 329

PfHsp70-x 358 DK[●]SQVHEIVLVGGSTRIPKIQQLK[●]DFN[●]GKEPC[●]KA[●]INPDEAVAYGA[●]AVQAA[●]ILSGDQSSAVK[●]DLLL 424
DnaK 330 SVSDID[●]VLVGGQTRMPMVQKKVAEFFGKEPRKDVNPDEAVAIGA[●]AVGGVLTGD----VKDVL 391

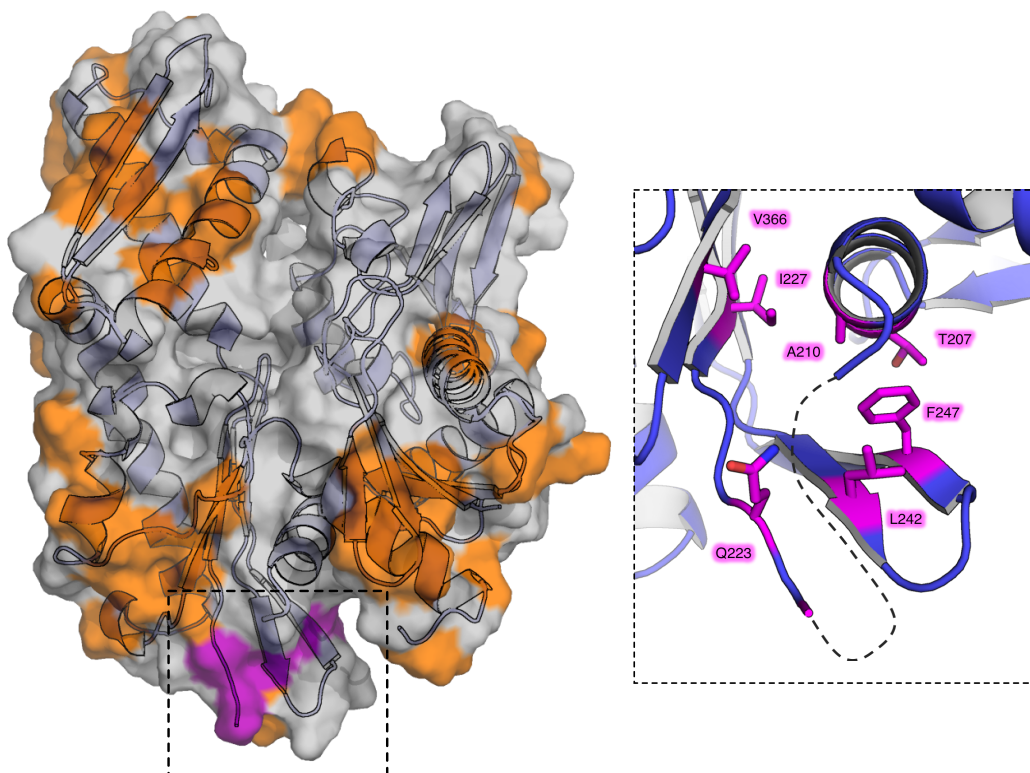


Figure 5.15: 115-7c predicted binding site. Pairwise alignment of PfHsp70-x and DnaK coloured by amino acid type and conservation. DnaK residues implicated in 115-7c binding and the corresponding PfHsp70-x residues are indicated by purple circles. The crystal structure of PfHsp70-x_{ATPase} in surface and cartoon representation. Residues corresponding to DnaK 115-7c binding are coloured pink on the PfHsp70-x_{ATPase} and non-conserved residues shown in orange.

<i>PfHsp70-x</i>	30	E	E	S	E	V	A	I	G	I	D	L	G	T	T	S	C	V	G	I	C	R	N	G	V	V	D	I	A	N	D	Q	G	N	R	T	T	P	S	Y	V	A	F	T	D	T	E	R	L	I	G	D	A	A	K	N	Q	A	S	R	N	P	E	N	T	95		
<i>Hsp72</i>	1	M	A	K	A	A	A	I	G	I	D	L	G	T	T	S	C	V	G	V	F	Q	H	G	K	V	E	I	A	N	D	Q	G	N	R	T	T	P	S	Y	V	A	F	T	D	T	E	R	L	I	G	D	A	A	K	N	Q	V	A	L	N	P	Q	N	T	66		
<i>PfHsp70-x</i>	96	V	F	D	A	K	R	L	I	G	R	K	F	S	E	T	T	V	Q	S	D	M	K	H	W	P	F	T	V	K	G	S	D	G	K	P	M	I	E	V	S	Y	Q	G	E	K	K	T	F	H	P	E	E	I	S	S	M	V	L	K	K	M	K	E	V	A	161	
<i>Hsp72</i>	67	V	F	D	A	K	R	L	I	G	R	K	F	G	D	P	V	V	Q	S	D	M	K	H	W	P	F	Q	V	I	N	D	G	-	D	K	P	K	V	Q	V	S	Y	K	G	E	T	K	A	F	Y	P	E	E	I	S	S	M	V	L	T	K	M	K	E	I	A	131
<i>PfHsp70-x</i>	162	E	T	Y	L	G	K	P	V	K	N	A	V	I	T	V	P	A	Y	F	N	D	S	Q	R	Q	A	T	K	D	A	G	A	I	A	G	L	N	V	L	R	I	N	E	P	T	A	A	A	I	A	Y	G	L	D	K	K	G	K	G	E	Q	N	I	L	I	227	
<i>Hsp72</i>	132	E	A	Y	L	G	Y	P	V	T	N	A	V	I	T	V	P	A	Y	F	N	D	S	Q	R	Q	A	T	K	D	A	G	V	I	A	G	L	N	V	L	R	I	N	E	P	T	A	A	A	I	A	Y	G	L	D	R	T	G	K	G	E	R	N	V	L	I	197	
<i>PfHsp70-x</i>	228	F	D	L	G	G	T	F	D	V	S	L	L	T	L	E	D	G	I	F	E	V	K	A	T	S	G	D	T	H	L	G	G	E	D	F	D	N	K	L	V	N	F	C	V	Q	D	F	K	K	K	N	G	G	K	D	V	S	K	N	S	K	S	L	R	R	293	
<i>Hsp72</i>	198	F	D	L	G	G	T	F	D	V	S	I	L	T	I	D	D	G	I	F	E	V	K	A	T	A	G	D	T	H	L	G	G	E	D	F	D	N	R	L	V	N	H	F	V	E	E	F	K	R	K	H	-	K	D	I	S	Q	N	K	R	A	V	R	R	262		
<i>PfHsp70-x</i>	294	L	R	T	Q	C	E	K	A	K	R	V	L	S	S	S	A	Q	A	T	I	E	V	D	S	L	F	D	G	I	D	Y	N	V	N	I	T	R	A	K	F	E	E	L	C	M	D	Q	F	R	N	T	L	I	P	V	E	K	V	L	K	D	A	K	M	D	K	359
<i>Hsp72</i>	263	L	R	T	A	C	E	R	A	K	R	T	L	S	S	S	T	A	S	L	E	I	D	S	L	F	E	G	I	D	F	Y	T	S	I	T	R	A	R	F	E	E	L	C	S	D	L	F	R	S	T	L	E	P	V	E	K	A	L	R	D	A	K	L	D	K	328	
<i>PfHsp70-x</i>	360	S	Q	V	H	E	I	V	L	V	G	G	S	T	R	I	P	K	I	Q	L	I	K	D	F	F	N	G	K	E	P	C	K	A	I	N	P	D	E	A	V	Y	G	A	A	V	Q	A	A	I	L	S	G	D	S	S	A	V	K	D	L	L	L	425				
<i>Hsp72</i>	329	A	Q	I	H	D	L	V	L	V	G	G	S	T	R	I	P	K	V	Q	K	L	L	I	K	D	F	F	N	G	R	D	L	N	K	S	I	N	P	D	E	A	V	Y	G	A	A	V	Q	A	A	I	L	M	G	D	K	S	E	N	V	Q	D	L	L	L	394	

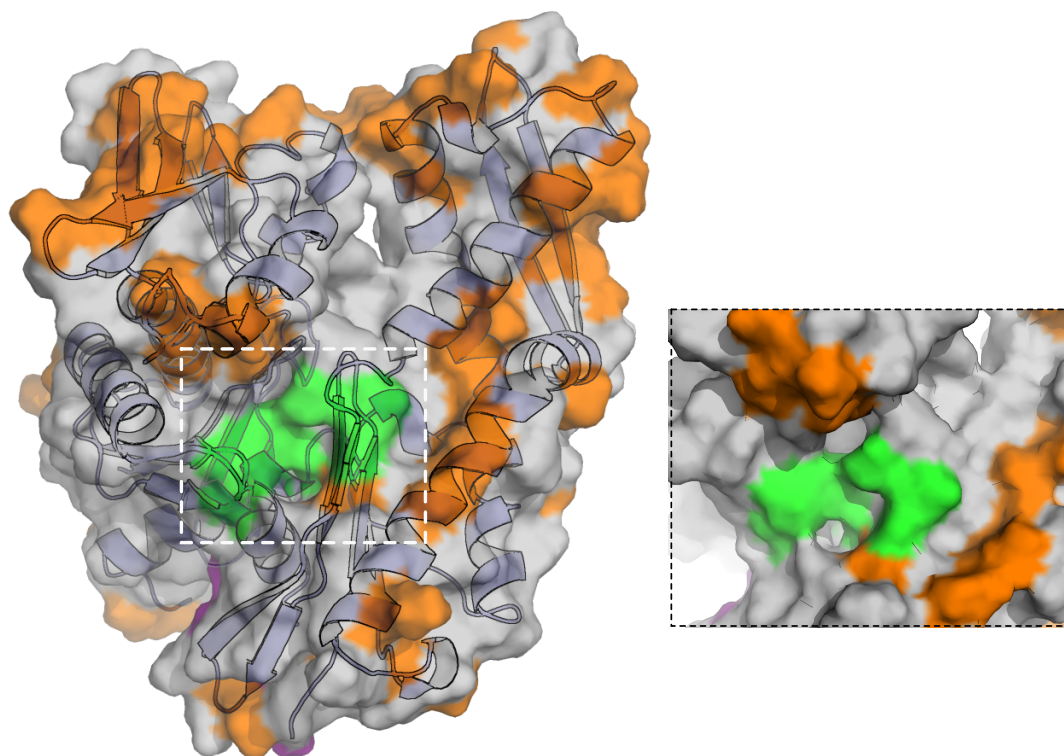


Figure 5.16: MKT-077 predicted binding site. Pairwise alignment of PfHsp70-x and Hsp72 coloured by amino acid type and conservation. Hsp72 residues implicated in MKT-077 binding and the corresponding PfHsp70-x residues are indicated by green circles. The crystal structure of PfHsp70-x_{ATPase} in surface and cartoon representation. Residues corresponding to Hsp72 MKT-077 binding are coloured green on the PfHsp70-x_{ATPase} and non-conserved residues shown in orange. Zoomed-in view of binding site shown in inset.

The rhodacyanine dyes, YM-01 and MKT-077, also inhibited PfHsp70-x ATPase activity. MKT-077 was discovered as an effective antitumour agent and was found to function via Hsp70s which play a crucial role in tumour proliferation. Subsequent studies demonstrated MKT-077 and its derivatives, including YM-01, bind to human Hsp72 in an allosteric pocket adjacent to the nucleotide binding site which prevents ATP hydrolysis (Fig. 5.16) [179]. A pairwise alignment of Hsc70 and PfHsp70-x reveals that these residues reside in areas of high conservation and that only one amino acid change (A223 -> S) can be seen. In fact, this pocket is highly conserved in general [178] and therefore explains why the compounds similarly inhibit PfHsp70-x. Mapping the pocket onto the PfHsp70-x structure, we can see it could be possible to design further MKT-077 derivatives that reach out of the pocket and interact with nearby patches of less-conserved residues to impart specificity of inhibition.

To conclude, we have shown that Hsp70 inhibitors are capable of inhibiting PfHsp70-x opening the door of repurposing Hsp70 inhibitors as antimalarials. Methylene blue is already in clinical trials and is proving to be an effective, safe and affordable treatment. It is a broadly acting antimalarial which reduces the chances of drug-resistance but knowing its targets and mechanism of action will be useful for the future in case of such resistance. We were also able to use the previously presented crystal structure of PfHsp70-x_{ATPase} (Chapter 4) to explain some differences seen in inhibition, such as that of 115-7c, and also to begin exploring how such inhibitors could be modified to impart specificity.

During our inhibition assays in which we also explored inhibition of the cochaperone stimulated activity of PfHsp70-x, we saw some interesting results; certain inhibitors were able to inhibit PfHsp70-x alone only or had preferential inhibition of PfHsp70-x in complex with a specific cochaperone. In the next chapter we aim to further understand the plasmodial cochaperones and their behaviour with PfHsp70-x and therefore explain the behaviour of the inhibitors assayed here.

6

PfHsp70-x and cochaperones PFA0660w and PFE0055c

Contents

6.1	The J domain of PFA0660w encodes specificity	126
6.2	Crystal structure of PFA0660w_J	127
6.3	Homology model of PFE0055c_J	135
6.4	The J domain - PfHsp70-x interaction	141
6.5	Discussion	152

In the previous chapter, we tested a number of commercially available Hsp70 inhibitors for their effect on PfHsp70-x. Inhibition of the cochaperone-stimulated activity of PfHsp70-x was also tested and revealed that some inhibitors behaved differently depending on if there was a cochaperone present or even which cochaperone was present such as methylene blue, 115-7c and 116-9e. Both methylene blue and 115-7c were less able to inhibit PFA0660w stimulated PfHsp70-x activity than PFE0055c stimulated. PFA0660w and PFE0055c are exported Hsp40s found to colocalise with PfHsp70-x in J-dots [71]. Previous studies revealed that PFA0660w specifically stimulates PfHsp70-x whereas it has been reported that PFE0055c is more promiscuous and can also stimulate the ATPase activity of human Hsp72 [91]. Specificity of Hsp40 proteins has been shown to be important in conferring

particular functions on Hsp70 chaperones by expanding their repertoire of client proteins [184]. PFA0660w may therefore allow PfHsp70-x to chaperone certain proteins that contribute towards pathogenicity.

In this chapter we aim to gain insights into the stimulation of PfHsp70-x by PFA0660w and PFE0055c to understand this specificity. Understanding the interaction of PfHsp70-x and its cochaperones may allow us to develop inhibitors effective against pathogenic Hsp70-Hsp40 complexes, thereby avoiding harmful effects through accidentally disrupting necessary functions of Hsp70s in the host.

6.1 The J domain of PFA0660w encodes specificity

Previous work studying the specific stimulation of PfHsp70-x by its cognate cochaperone PFA0660w were performed using full-length PFA0660w [91]. Here we study the cochaperone specificity of the J domain alone as the full-length protein is not amenable to crystallography due to disordered, flexible regions, nor to NMR due to large molecular weight of the full-length Hsp40 dimer. It is widely believed that the J domain is sufficient for stimulation of Hsp70s. However, the basis and localisation of Hsp40 specificity still remains somewhat a mystery: some J domain swapping experiments were successful in changing specificity [185, 186], but others were not [187].

The ATP hydrolysis activity of both PfHsp70-x and Hsc70, basal and stimulated, were measured using the MESG assay described in the previous chapter. PFA0660w_J and PFE0055c_J were added at varying concentrations to determine the degree of cochaperone stimulation (Fig. 6.1). Stimulation by full-length *E.coli* DnaJ, a promiscuous Hsp40, was also measured to compare rates of stimulation of full-length and J domain constructs.

We can see that PfHsp70-x ATP hydrolysis activity is stimulated at similar levels by PFA0660w_J and PFE0055c_J (stimulated activity to 1.8 fold and 2.2 fold at 10 x molar excess, respectively). Consistent with data for full-length PFA0660w and PFE0055c with Hsp72, we see that PFA0660w_J is unable to stimulate Hsc70

activity except at very high concentrations of 250 μM (50 x molar excess) whereas PFE0055c is able to stimulate Hsc70, albeit at a lesser degree than for PfHsp70-x (1.3 fold stimulation at 10 x molar excess). Therefore, we can confirm that the J domain encodes the specificity of PFA0660w.

The degree of stimulation by the J domains alone is far lower than that caused by full-length DnaJ. We can see that for both PfHsp70-x and Hsc70, stimulation reaches 14 fold and 10 fold, respectively, at the highest concentrations of DnaJ whereas stimulation reaches a maximum at 2 – 2.5 fold for both J domains. This is consistent with previous studies in which full-length PFA0660w doubled the ATPase activity of PfHsp70-x at just a 1:2 molar ratio of chaperone to cochaperone demonstrating the superior stimulation of full-length compared to J domain alone. The reduction in stimulation is likely due to the ability of type I and type II Hsp40s to dimerise forming a central cleft to which the substrate binding domain of Hsp70s docks [188].

To understand the contrasting behaviours of PFA0660w and PFE0055c, we aimed to structurally characterise the J domain of the parasitic cochaperones. A number of crystal trials were set up for both PFA0660w_J and PFE0055c_J at various protein concentrations. Attempts at creating diffracting crystals of PFE0055c_J were unfortunately unsuccessful, however we were able to obtain PFA0660w_J crystals.

6.2 Crystal structure of PFA0660w_J

PFA0660w_J crystals diffracted to 1.39 Å and the structure was readily solved by molecular replacement using the crystal structure of the J domain of the yeast Hsp40, Sis1 (PDB 4RWU) as the template model and Refmac [189]. Autobuild [114] was able to place the majority of the amino acid sequence of PFA0660w_J and the remaining residues were built manually using Coot [113]. Refinement was carried out using Phenix.Refine [190] iteratively in cycles alongside building in Coot. Statistics of data collection and structure refinement can be seen in Table 6.1.

The asymmetric unit contains two almost identical copies of PFA0660w_J (all-atom RMSD 0.3 Å) organised in an A-like shape (Fig. 6.2). The two copies interact through a small interface consisting of 7 salt bridges and 2 hydrogen bonds largely

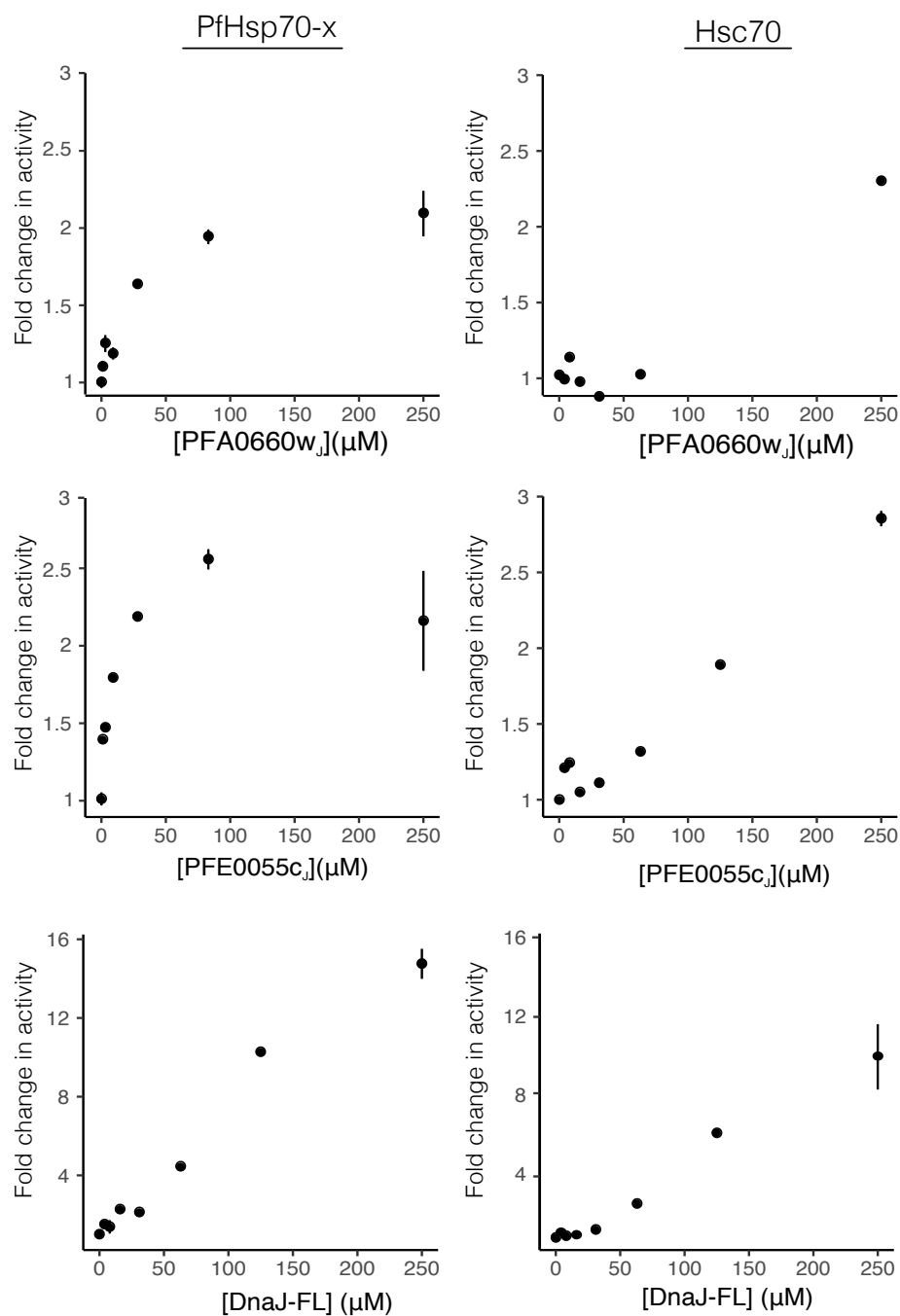


Figure 6.1: Stimulation of PfHsp70-x and Hsc70 ATPase activity by PFA0660w_J, PFE0055c_J and DnaJ-FL. Number of replicates = 3. Error bars represent standard error of fitting raw time-course data to a linear model.

between K100, D93, R96 and D86 with some contributions from the side chain of S90 and backbone atoms of G84. The biological importance of this interface was assessed by PISA on the basis of interface area, solvation energy, number of bonds and total binding energy and found to not be biologically significant but an artefact of crystallization [145].

Examining the structure of PFA0660w_J, we can see that the domain is comprised of 4 α -helices that pack tightly together joined by loops of varying lengths (Fig. 6.3). Helices II and III lay antiparallel to each other with the short helix I running across one side. Helix IV projects from helix III at roughly 90° towards the same face occupied by helix I. Interestingly, helix II has an interruption towards the C-terminal end causing the final turn of the helix to be projected away from the protein. On this unusual region lies the HPD motif: a highly conserved sequence of three residues (histidine, proline and aspartate) previously shown to be necessary for Hsp70 activity stimulation [191]. The additional flexibility expected from this disrupted portion of helix may allow the HPD motif to reach further into clefts for stimulation of PfHsp70-x.

A number of J domain structures have been previously determined by X-ray crystallography or NMR. J domains from a variety of organisms (bacterial to fungal, mammalian to nematode) are shown alongside the structure of PFA0660w_J demonstrating the conservation of the domain's fold (Fig. 6.4). All shown structures have two long helices laying antiparallel joined by either a long linker (*E. coli* DnaJ [192], *C. elegans* Dnj-12 [193] and human Hsp40 [194]) or a shorter linker with a disrupted helix (*P. falciparum* PFA0660w and *B. Taurus* Auxilin [195]) containing the highly conserved HPD motif. The HPD motif seems to always localise to this flexible region implying that the ability to move independently from the rest of the protein is integral to its role in stimulation, perhaps through an induced fit mechanism. In most structures, two additional short helices are present across one face of the antiparallel helices, such as is seen in PFA0660w, stabilising the positions of helices II and III.

Table 6.1: Table of data collection and refinement statistics for the *PFA0660w_J* crystal structure.

Protein	<i>PFA0660w_J</i>
Space group	P 1 2 ₁ 1
Unit cell (Å, °)	25.83 54.93 51.44, 90 98.2 90
Beamline	DLS/I24
Wavelength (Å)	1.0389
Resolution range (Å)	37.34 – 1.38
High-resolution shell (Å)	(1.43 – 1.38)
R _{merge} (I)	0.071 (0.600)
R _{meas} (I)	0.085 (0.728)
R _{pim} (I)	0.049 (0.405)
Completeness (%)	97.6 (99.4)
Multiplicity	3.3 (3.1)
I/σ	9.48 (2.04)
CC ½	0.99 (0.52)
Wilson B factor	12.73
Refinement statistics	
R _{work} (reflections)	0.1647 (28671)
R _{free} (reflections)	0.2027 (1335)
Number of atoms	
Protein	1140
Water	124
Average B factors (Å²)	
Protein	17.1
Water	29.3
RMSD from ideal	
Bonds/angles (Å / °)	0.009/1.29
MolProbity statistics	
Ramachandran favored (%)	100
Ramachandran disallowed (%)	0
Ramachandran outliers (%)	0
Rotamer outliers (%)	0
Clashscore (percentile)	0.88 (99 th)
MolProbity score (percentile)	0.77 (100 th)

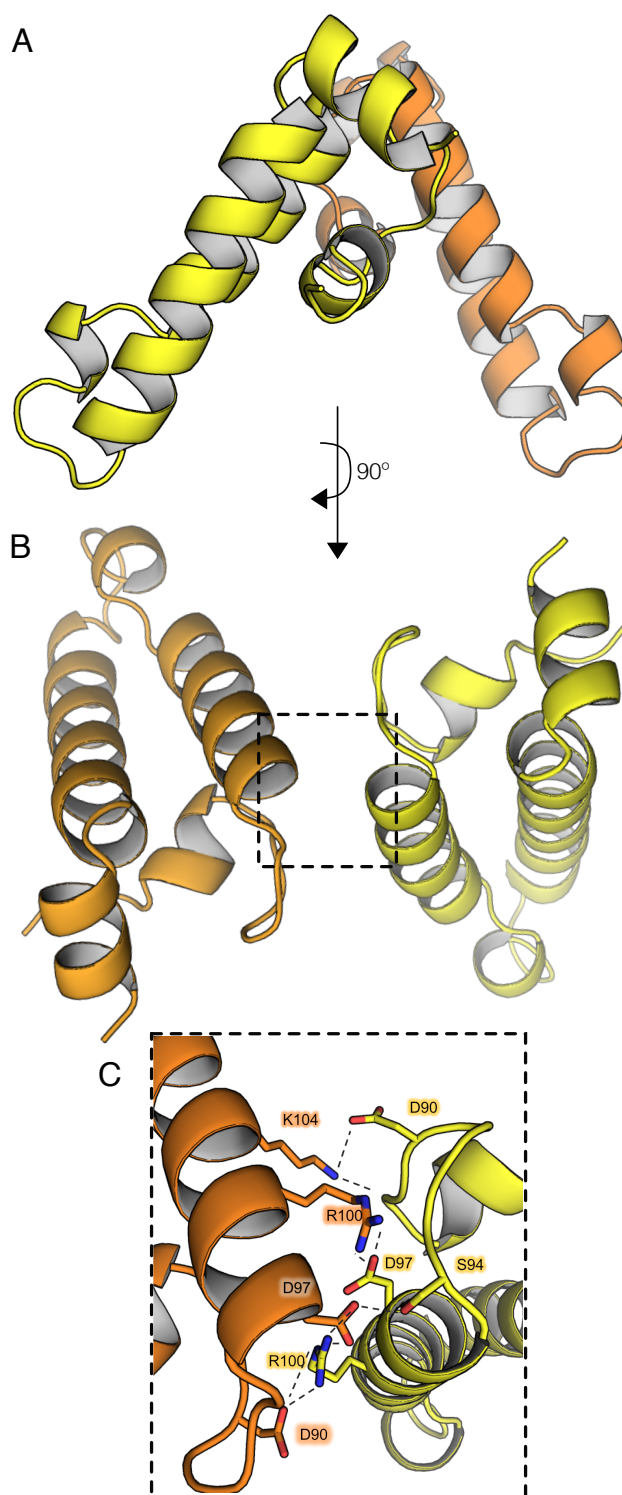


Figure 6.2: Asymmetric unit of PFA0660w_J crystal structure. **A:** Asymmetric unit view from the side. Copy A is shown in yellow and copy B in orange. **B:** Asymmetric unit as shown from above. **C:** Detailed view of the interface between copies as indicated by the dashed box in B. Interacting residues are shown in stick representation and interactions indicated by dashed lines.

The J domain of PFA0660w is similar in overall fold to that of other Hsp40 proteins leaving the question of specificity unanswered. To understand the basis of differential specificity between PFA0660w and PFE0055c we performed a pairwise alignment using EMBOSS on the EMBL server [180] and mapped sequence differences onto the structure of PFA0660w_J (Fig. 6.5). The pairwise alignment of PFA0660w and PFE0055c show a high degree of conservation within the J domain. We see that non-conserved residues are spread throughout the sequence of PFA0660w_J and present on all helices. The majority of differences appear on the ‘front’ face of helices II and III but the C-terminal half of helix II, containing the HPD motif, remains identical between the two cochaperones. Of particular interest is residue L103 on helix II which is an arginine in PFE0055c. It has been previously shown that mutation of the corresponding arginine residue in bacterial DnaJ’s significantly affected Hsp70 binding and stimulation, thereby perhaps explaining PFA0660w’s inability to stimulate other Hsp70s [196, 197]. It is still unclear how PFA0660w is able to stimulate the activity of PfHsp70-x however.

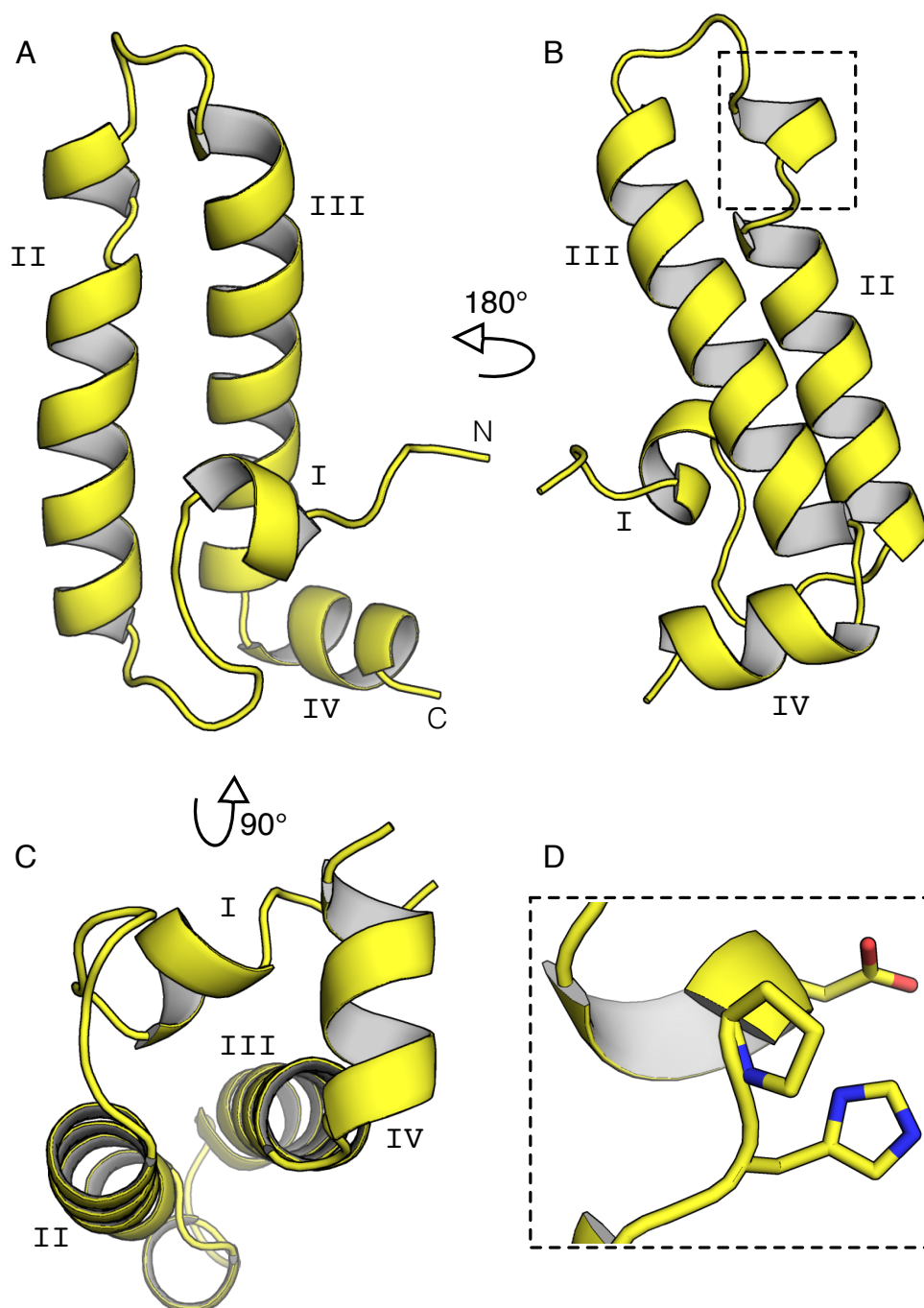


Figure 6.3: The crystal structure of PFA0660w_J. **A:** PFA0660w_J crystal structure with helices numbered I-IV from N- to C-terminus. **B:** **A** rotated by 180° along the y axis. **C:** **A** rotated by 90° along the x axis. **D:** A zoomed-in view of the HPD motif projecting away from the protein. The zoomed region is indicated by the dashed box in **B**.

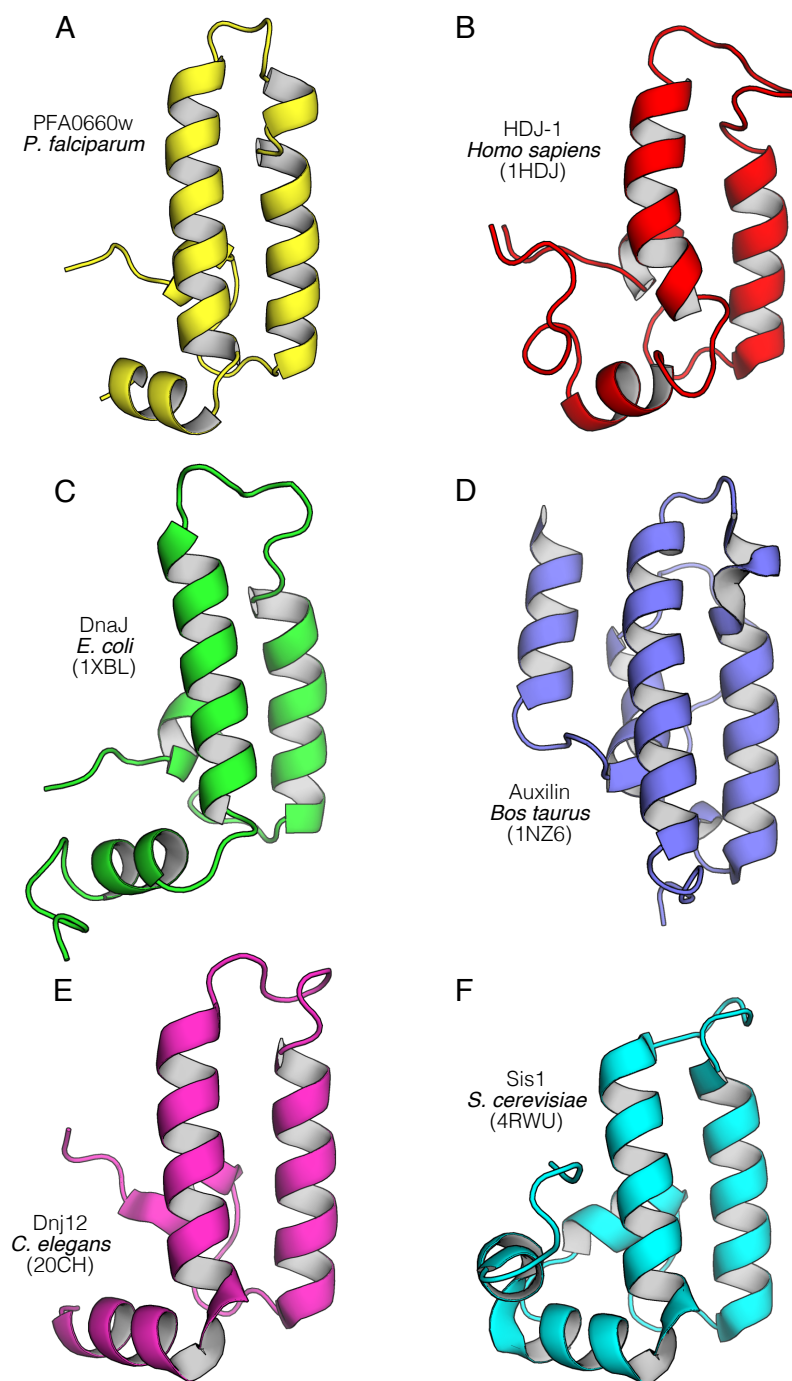


Figure 6.4: A comparison of J domain structures. **A:** Crystal structure of PFA0660w_J. **B:** NMR structure of the J domain of human Hsp40, HDJ-1 (PDB:1HDJ) [194]. **C:** NMR structure of *E. coli* DnaJ J domain (PDB:1XBL) [192]. **D:** Crystal structure of Auxilin J domain from *Bos taurus* (PDB:1NZ6) [195]. **E:** Crystal structure of Dnj12 J domain from *C. elegans* (PDB:20CH) [193]. **F:** Crystal structure of Sis1 J domain from *S. cerevisiae* (PDB:4RWU) [198].

A

<i>PFA0660w</i>	76	FGKSSMDYYTLLGV	DKGCS	EDDLRRAYL	KLAMKWHPDKHVN	116
<i>PFE0055c</i>	74	SKTDETDYYAVLGLT	KDCTQDD	IKKAYRKLAMKWHPDKHLN		114
<i>PFA0660w</i>	117	KGSKVEAEKFKN	ICEAYS	VLSDNEKRVKYDLFG		148
<i>PFE0055c</i>	115	DEDKVEAERKFKL	IGEAYE	VLSDEEKRNKYDLFG		150

B

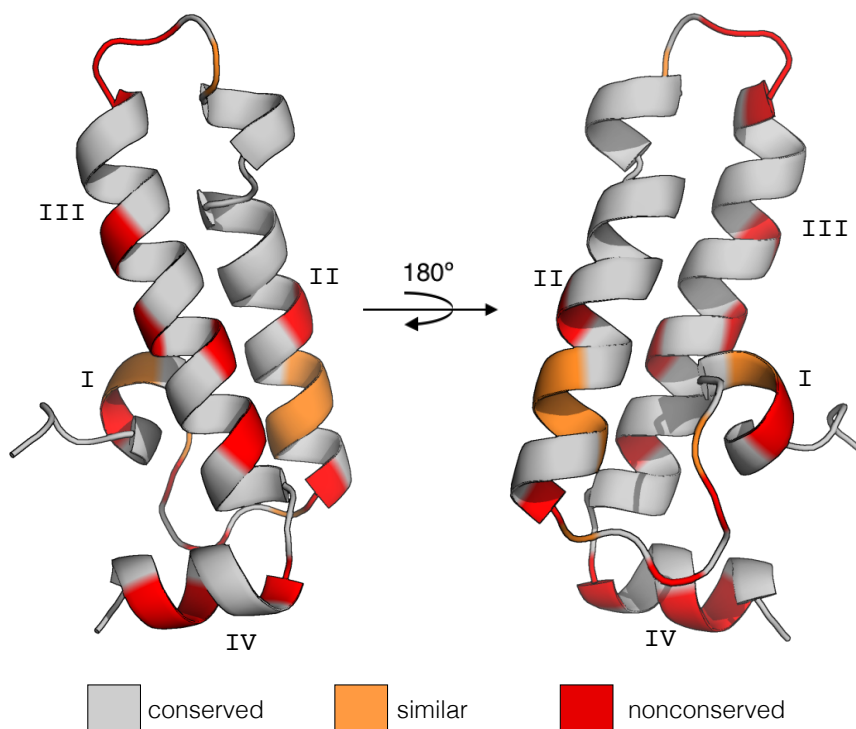


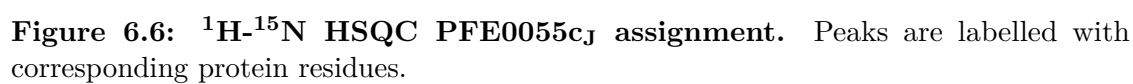
Figure 6.5: Mapping J domain similarities. **A:** A pairwise alignment of *PFA0660w* and *PFE0055c* J domains performed using EMBOSS [180] and coloured by conservation. **B:** Mapping of similar and non-conserved residues onto the structure of *PFA0660w_J*. Conserved residues are shown in grey, similar shown in orange and non-conserved in red.

6.3 Homology model of *PFE0055c_J*

As attempts at crystallizing *PFE0055c_J* were unsuccessful and the sequence similarity of *PFE0055c_J* and *PFA0660w* is high, we generated a homology model of *PFE0055c_J* using the SWISS-MODEL server [199]. *PFA0660w_J* is the homologue with the highest sequence similarity and a solved structure so it was used as the template onto which the sequence of *PFE0055c* was threaded. The resulting homology model had a Global Model Quality Estimation (GMQE) score of 0.86 out of 1, with scores approaching 1 indicating higher reliability.

Our PFE0055c_J homology model was validated by the protein energetic conformational analysis (PECAN) server, which combines amino acid sequence information with NMR chemical shifts to determine elements of secondary structure (Fig. 6.7) [121]. To do so, first we performed sequential backbone assignments to correlate peaks in the ¹H-¹⁵N HSQC spectrum to residues in the protein (Fig. 6.6). All PFE0055c_J residues were assigned to except for D95, P109 and K140. We can see that the PFE0055c_J homology model has very similar secondary structure elements as that predicted by PECAN, which suggests that the homology model is representative of the solution structure (Fig. 6.7).

Validation was also performed for the crystal structure of PFA0660w_J to confirm the structure is representative of that in solution and to demonstrate the accuracy of the protein energetic conformational analysis. A sequential backbone assignment of PFA0660w_J was also performed and all residues except P111 were assigned (Fig. 6.8). PECAN analysis predicts secondary structure elements almost identical to those seen in the crystal structure, even the disrupted section of helix II, confirming the crystal structure to be representative of the solution state (Fig. 6.9).



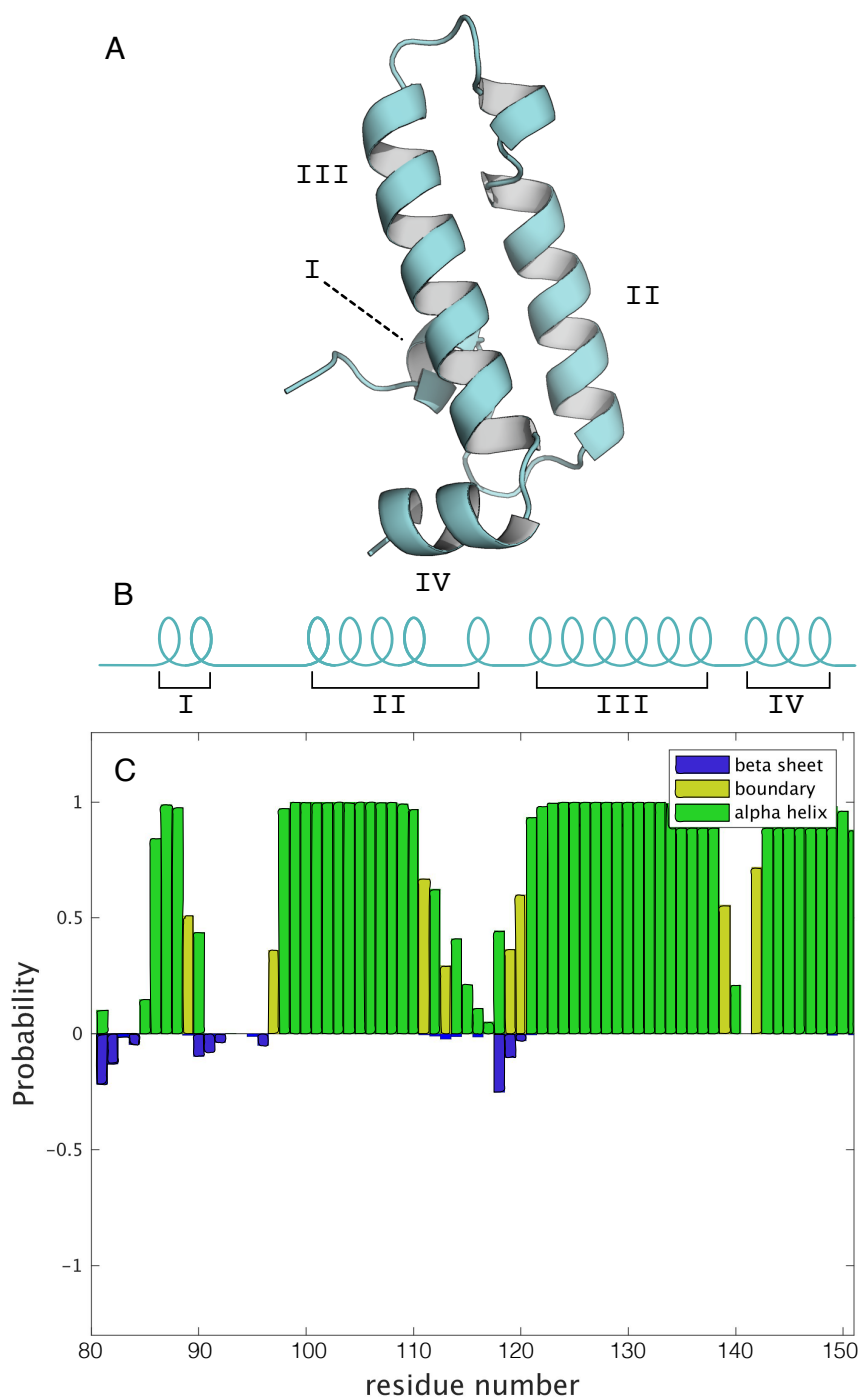


Figure 6.7: Secondary structure analysis of PFE0055c_J using PECAN. **A:** Homology model of PFE0055c_J generated by SWISS-MODEL with helices numbered I-IV. **B:** Representation of secondary structure in the homology model. **C:** PECAN analysis indicating regions and types of secondary structure. Probability of the type of secondary structure is plotted for each residue and coloured according to type of secondary structure.

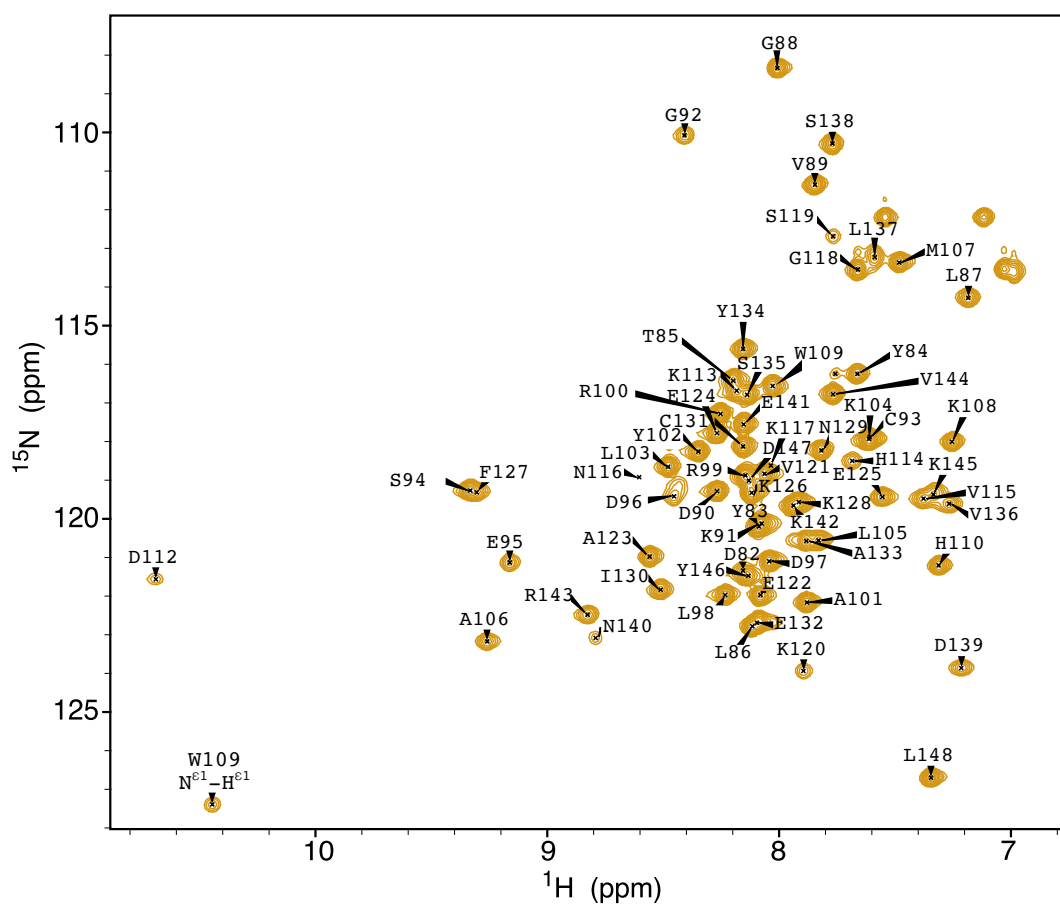


Figure 6.8: ^1H - ^{15}N HSQC *PFA0660w_J* assignment. Peaks are labelled with corresponding protein residues.

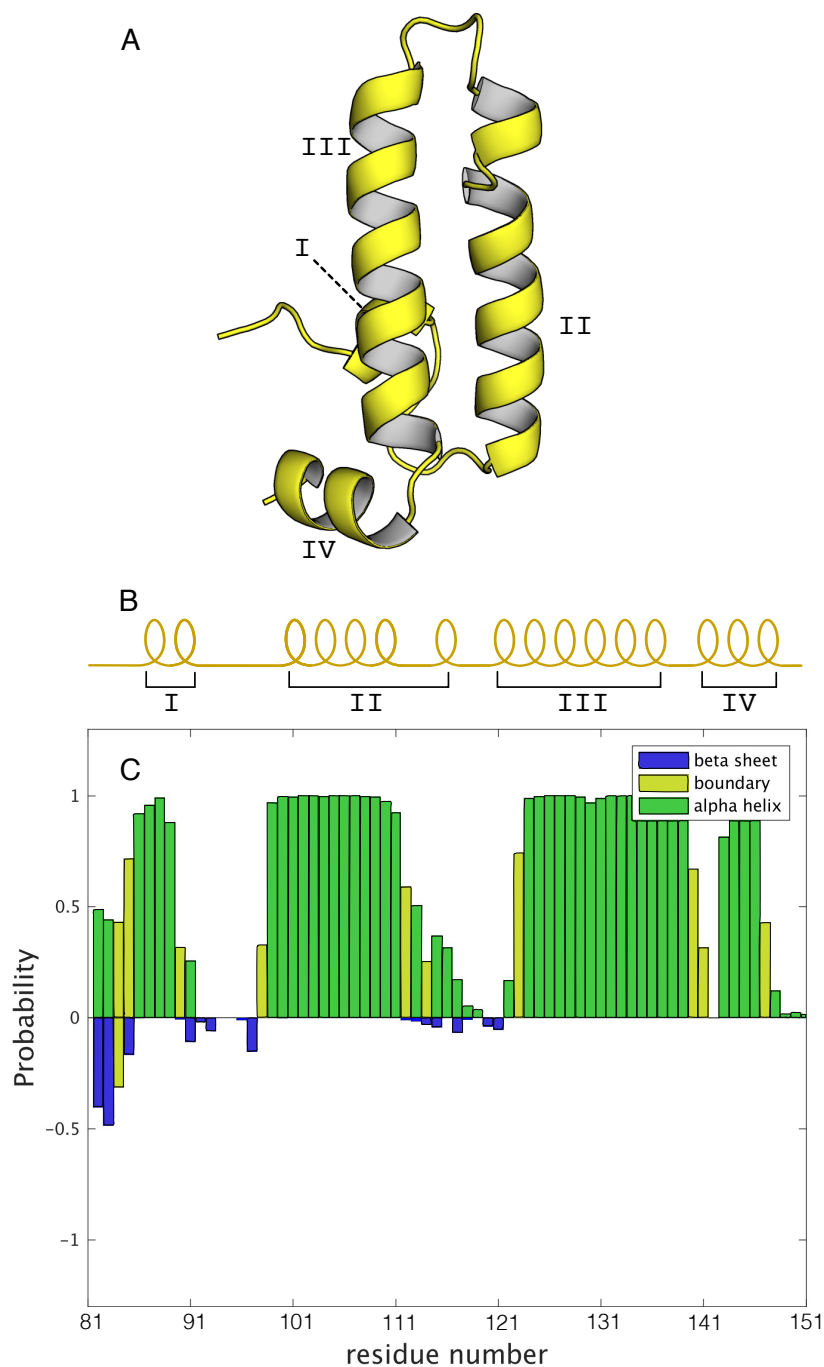


Figure 6.9: Secondary structure analysis of PFA0660w_J using PECAN. **A:** Crystal structure of PFA0660w_J with helices numbered I-IV. **B:** Representation of secondary structure in the crystal structure. **C:** PECAN analysis indicating regions and types of secondary structure. Probability of the type of secondary structure is plotted for each residue and coloured according to type of secondary structure.

6.4 The J domain - PfHsp70-x interaction

To understand the differences in PFA0660w_J and PFE0055c_J binding to the Hsp70s, we used ¹H-¹⁵N HSQC titrations to determine the residues most affected on the J domains upon PfHsp70-x binding. Spectra were recorded for ¹⁵N labelled PFA0660w_J (Fig. 6.10) or PFE0055c_J (Fig. 6.11) alone in the presence of either PfHsp70-x_{FL} or Hsc70_{FL} at a molar ratio of 1:5 in the absence of any nucleotide. Upon overlaying spectra, we can see that the presence of the chaperone causes a decrease in peak intensity indicating that the interaction is in the μ s - ms time regime. Certain peaks experience a greater reduction in intensity than others indicating they are more affected by addition of chaperone. It is possible to see differences already between addition of PfHsp70-x or Hsc70 to PFA0660w_J. For example, the intensity of peaks corresponding to L103 and Y102 are more greatly reduced in the presence of PfHsp70-x than with Hsc70 (Fig. 6.12). We can also see there is a greater reduction in peak intensity overall when in the presence of PfHsp70-x compared to Hsc70, suggesting PFA0660w_J has a tighter affinity for PfHsp70-x.

To quantify these changes, the reduction in peak intensities were measured in each of the spectra and the mean percentage change was calculated (Fig. 6.13). Residues with percentage change values more than one standard deviation above the mean were deemed to be significantly affected by chaperone binding. Significantly affected residues were mapped onto the crystal structure and homology model of PFA0660w_J and PFE0055c_J, respectively (Fig. 6.14).

Initially, looking at PFA0660w_J in the presence of both PfHsp70-x_{FL} and Hsc70_{FL}, we can see that residues significantly affected lay exclusively on helix II. We can see more residues are affected in the presence of PfHsp70-x_{FL} than with Hsc70 which is consistent with the different degrees of stimulation observed for these Hsp70s, as a larger number of residues involved in binding may translate to tighter binding. Interestingly, the HPD motif does seem to play a part in PFA0660w_J's interaction with both PfHsp70-x and Hsc70 although it is not able to stimulate Hsc70. This is surprising, as the HPD motif has been previously shown to be necessary for Hsp70 stimulation by Hsp40s; thus one would expect PFA0660w_J to be capable

of stimulating Hsc70 as the HPD motif seems to be involved in binding. D112 of the HPD motif is not shown to be significantly affected by PfHsp70-x binding but is by Hsc70 binding, perhaps explaining the disparity in stimulation.

It should also be noted that L103, which was previously discussed to be of interest as this residue is usually a necessary arginine, is involved in the binding of PfHsp70-x but not seen to be affected in the presence of Hsc70. Alongside L103, we see Y102 is also affected by PfHsp70-x but not by Hsc70 suggesting that PFA0660w_J may be interacting with a hydrophobic patch on PfHsp70-x that is not present on other chaperones leading to increased affinity and specificity.

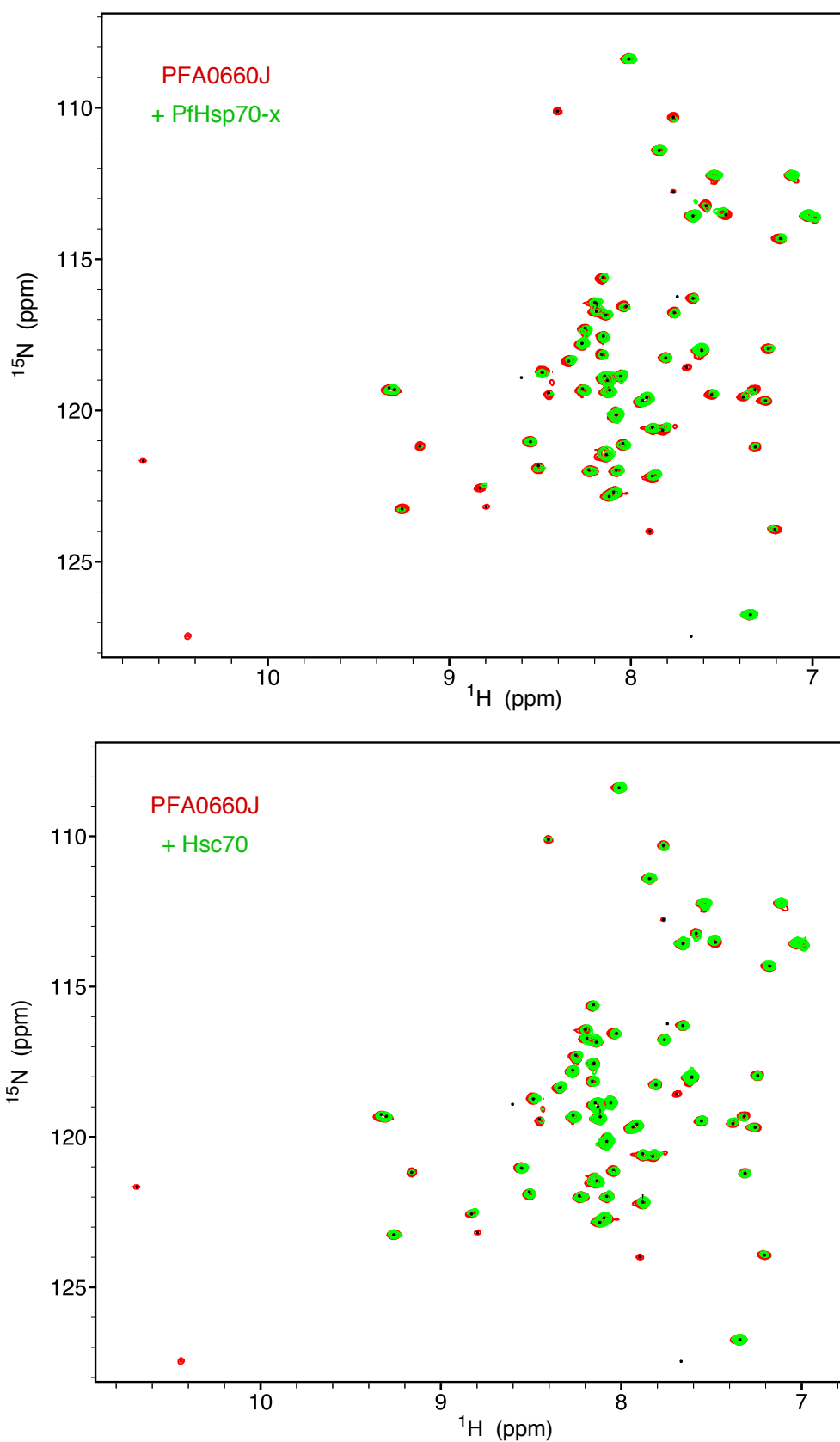


Figure 6.10: NMR of PFA0660w_J with PfHsp70-x and Hsc70. Overlaid spectra of PFA0660w_J (red) and PFA0660w_J with either PfHsp70-x or Hsc70 (green).

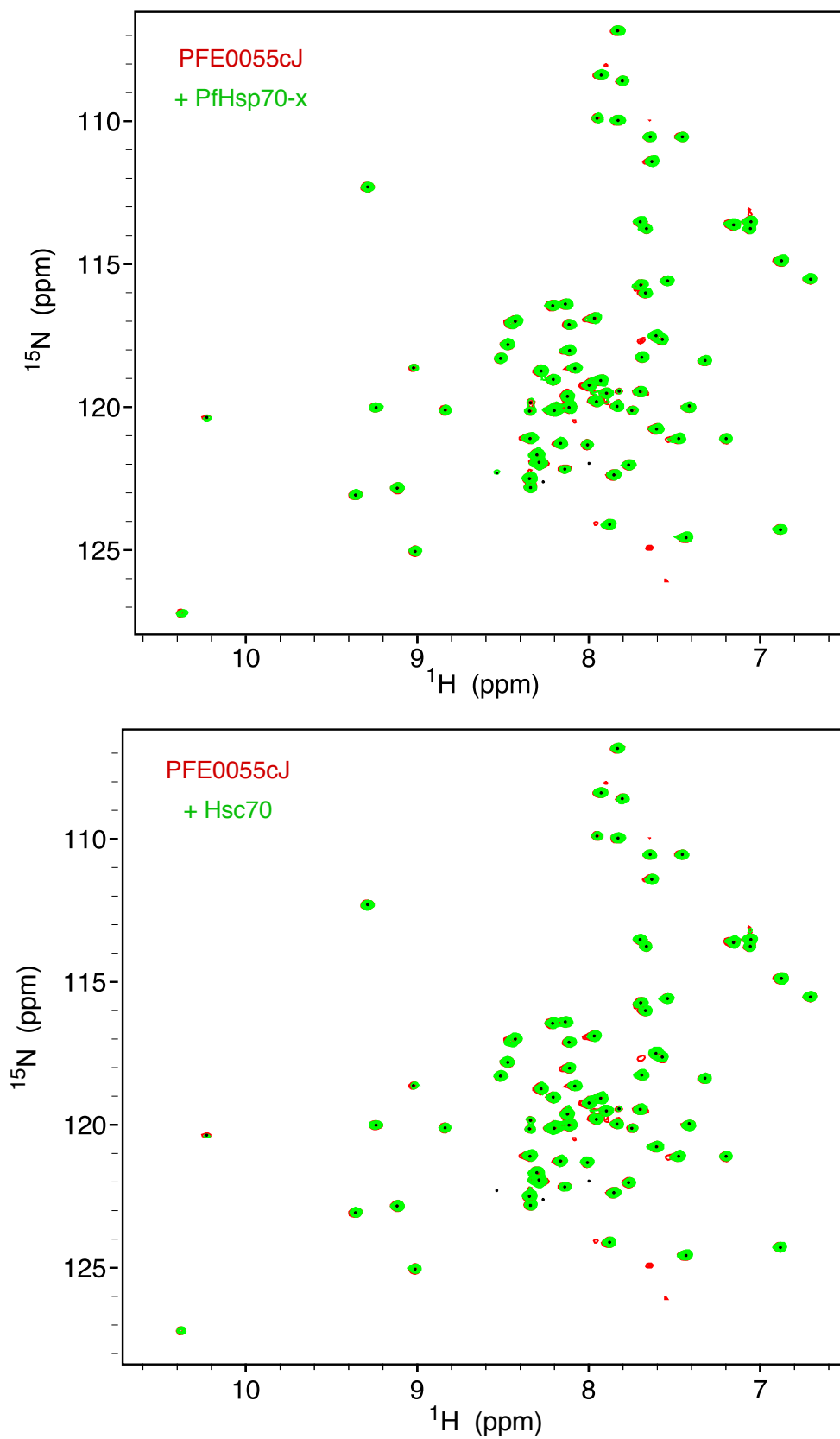


Figure 6.11: NMR of PFE0055cJ with PfHsp70-x and Hsc70. Overlaid spectra of PFE0055cJ (red) and PFA0660wJ with either PfHsp70-x or Hsc70 (green).

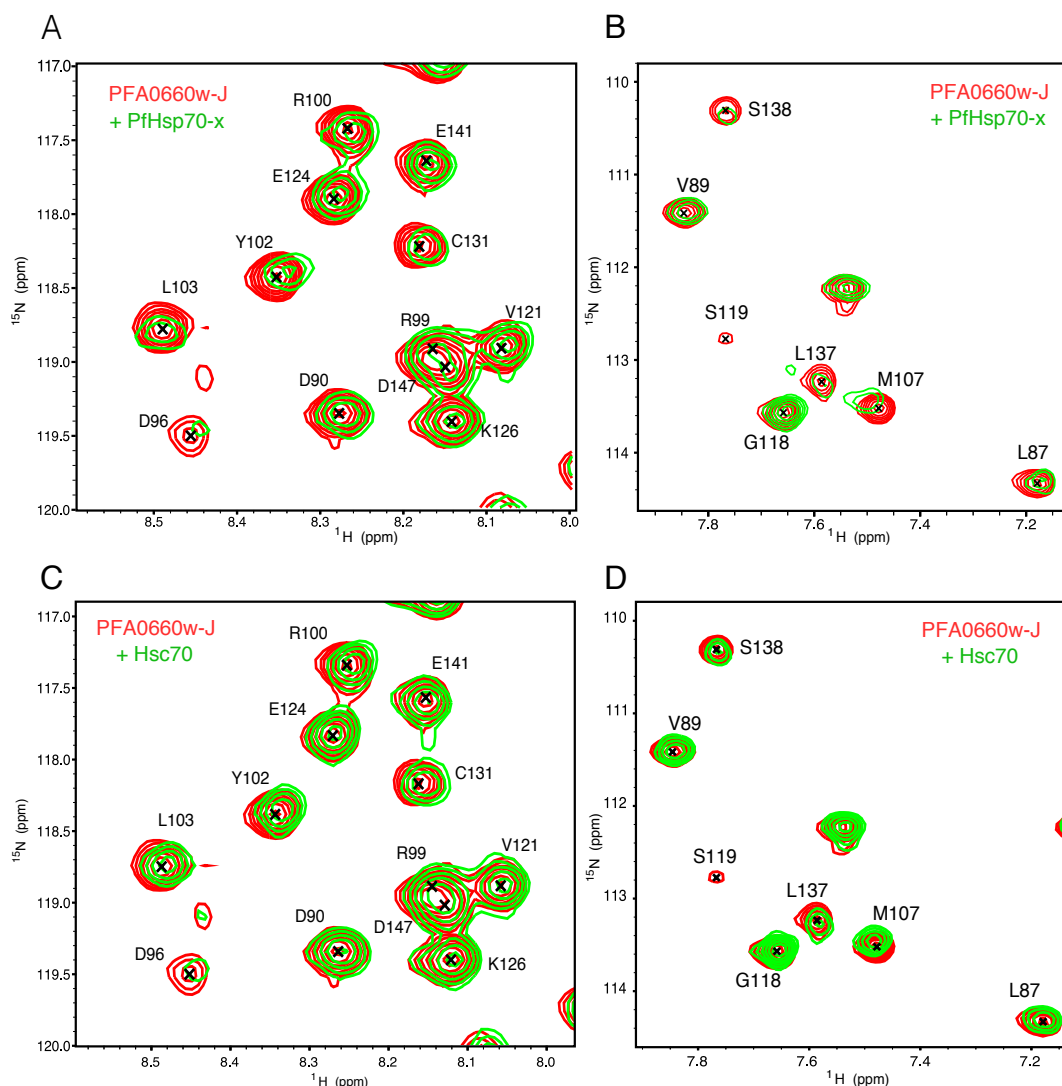


Figure 6.12: NMR titrations of cochaperones with chaperones. **A:** A close up of overlaid spectra PFA0660w_J alone (red) and in the presence of PfHsp70-x (green). Peaks are labelled with corresponding residues. **B:** Another region of overlaid spectra PFA0660w_J alone (red) and in the presence of PfHsp70-x (green). **C:** A close up of overlaid spectra PFA0660w_J alone (red) and in the presence of Hsc70 (green). **D:** Another region of overlaid spectra PFA0660w_J alone (red) and in the presence of PfHsp70-x (green).

Next, we examine the most affected residues of PFE0055c_J in the presence of PfHsp70-x and Hsc70, both of which can be stimulated by PFE0055c. Instantly, we can see that more residues are significantly affected by both PfHsp70-x and Hsc70 binding than was the case for PFA0660w_J. Further, the PFE0055c_J residues affected are similar whether PfHsp70-x or Hsc70 is present. Unlike PFA0660w_J,

the residues significantly affected are spread throughout helices I, II and III as well as on the front and back faces of the protein. Interestingly, we see neither the HPD motif nor R101 affected by the presence of PfHsp70-x or Hsc70 which is unusual as previous studies have shown these residues in other Hsp40s to be necessary for chaperone stimulation. Of particular note is the sequence ERKF on helix III as this is EEKF in PFA0660w – perhaps the change of R > E in this motif prevents PFA0660w from generally binding Hsp70s and therefore not being able to stimulate their activity. Another region of interest is the buried hydrophobic patch between helices I, II and III (Fig. 6.15) in PFE0055c_J which we see to be significantly affected by PfHsp70-x and, to a lesser extent, Hsc70 binding. As this patch is buried, it is unlikely that the residues are directly interacting with the chaperone but may instead undergo a slight rearrangement. This area is not seen to be similarly affected in PFA0660w binding to PfHsp70-x or Hsc70.

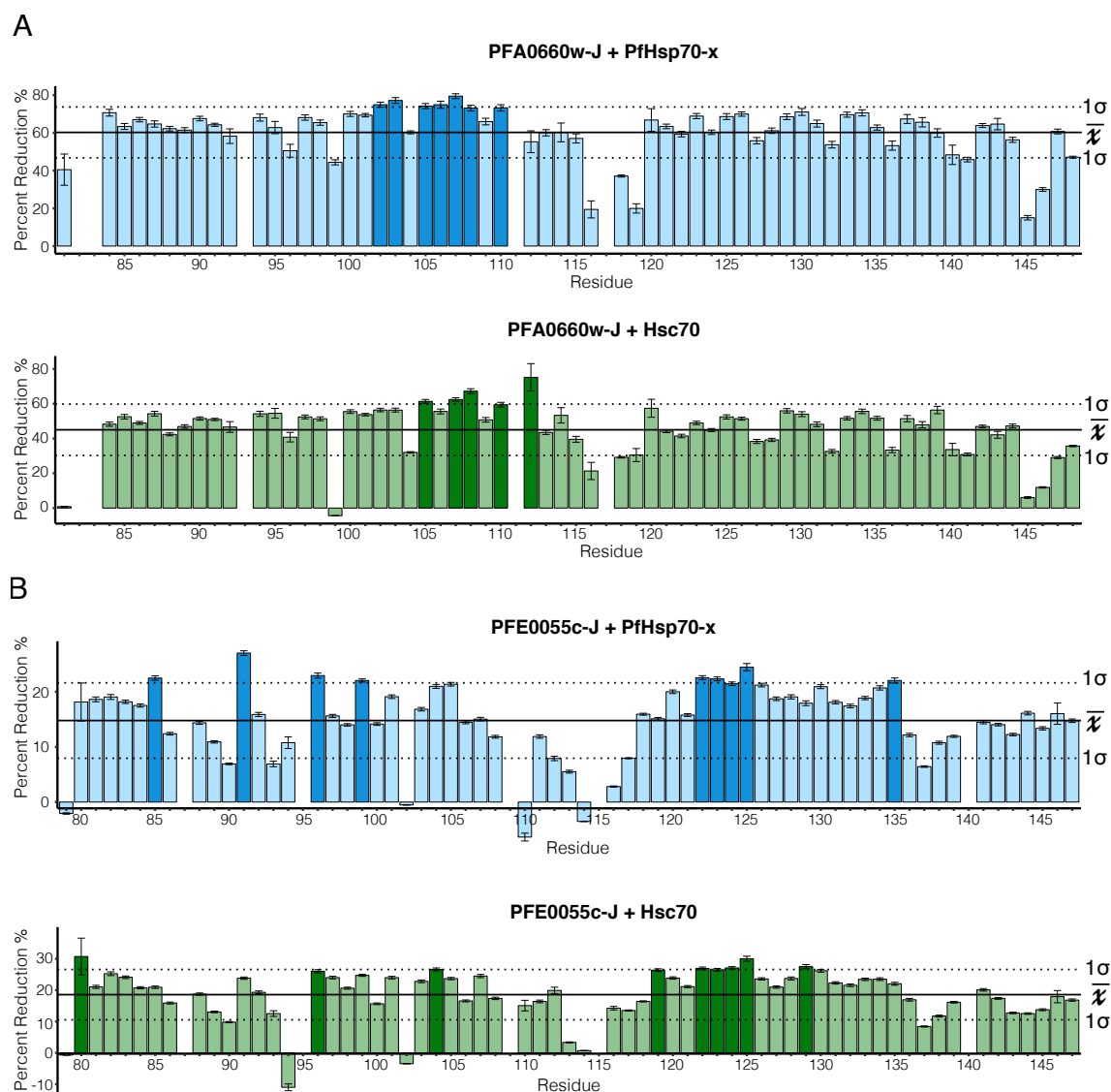


Figure 6.13: Peak intensity reduction by residue. **A:** A bar chart plotting percent reduction of peak intensity per residue of PFA0660w_J upon addition of PfHsp70-x (top) or Hsc70 (bottom). Residues deemed to be significantly reduced (above the 1 standard deviation threshold) are coloured darker. Mean percent reduction ($\bar{x}$) is indicated by a solid line and one standard deviation from the mean (1σ) is indicated by dashed lines. **B:** A bar chart plotting percent reduction of peak intensity per residue of PFE0055c_J upon addition of PfHsp70-x (top) or Hsc70 (bottom). Residues deemed to be significantly reduced (above the 1 standard deviation threshold) are coloured darker. Mean percent reduction ($\bar{x}$) is indicated by a solid line and one standard deviation from the mean (1σ) is indicated by dashed lines.

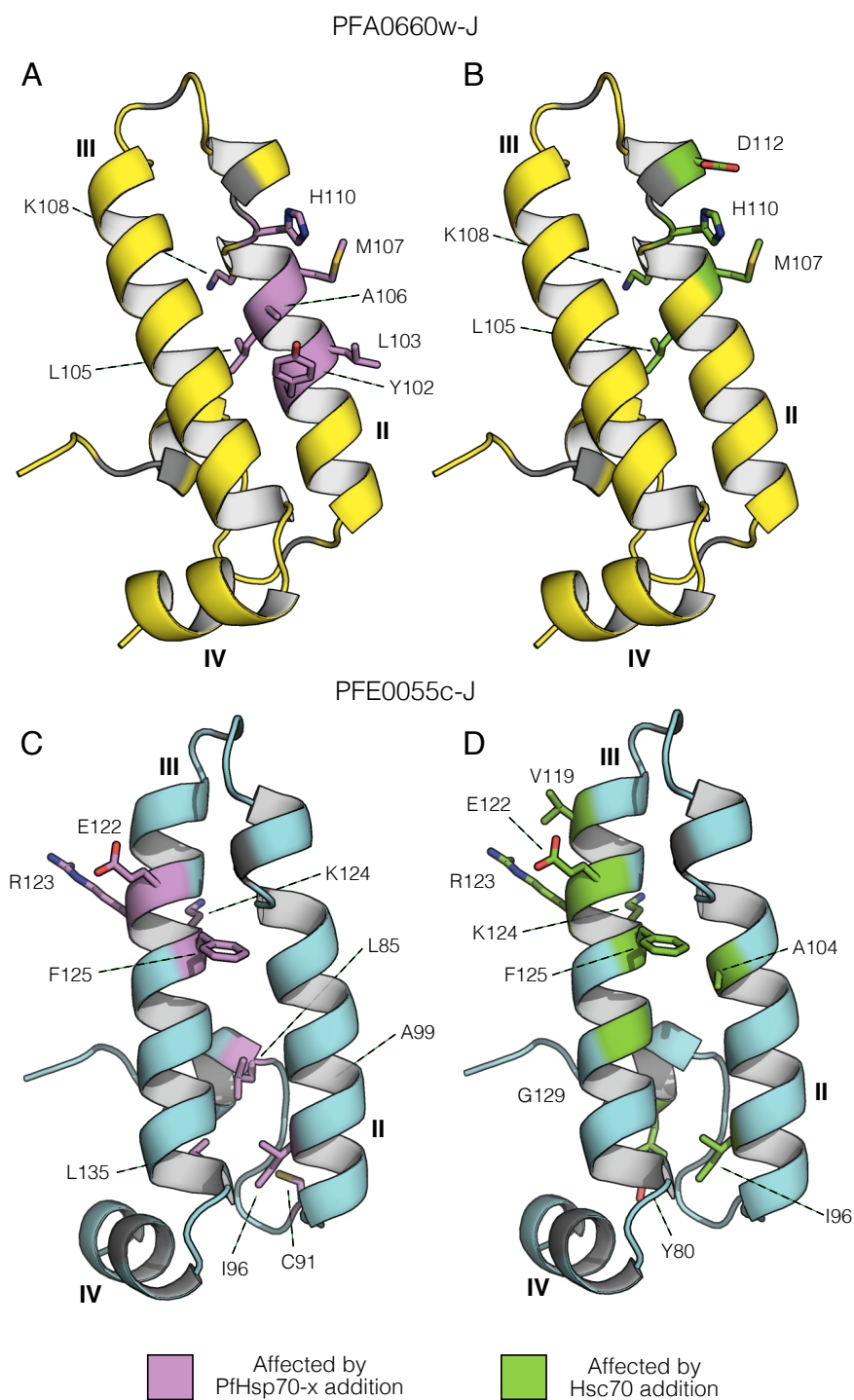


Figure 6.14: Mapping perturbations onto J domain structures. **A:** Crystal structure of PFA0660w_J with residues significantly affected by addition of PfHsp70-x highlighted in purple and shown in stick representation. **B:** Crystal structure of PFA0660w_J with residues significantly affected by addition of Hsc70 highlighted in green and shown in stick representation. **C:** Homology model of PFE0055c_J with residues significantly affected by addition of PfHsp70-x highlighted in purple and shown in stick representation. **D:** Homology model of PFE0055c_J with residues significantly affected by addition of Hsc70 highlighted in green and shown in stick representation.

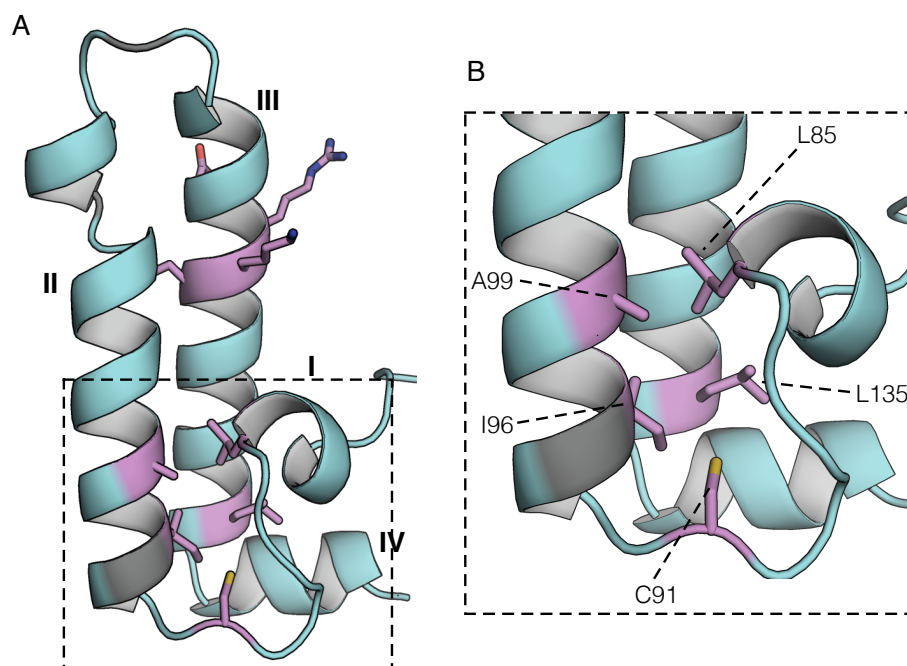


Figure 6.15: The hydrophobic patch of PFE0055c_J. **A:** Homology model of PFE0055c_J as seen from behind. Residues significantly affected by PfHsp70-x binding are shown in stick representation and coloured purple. Shown in grey are unassigned residues. The dashed box indicates the buried hydrophobic residues shown in detail in **B**: A detailed view of the hydrophobic residues affected by PfHsp70-x binding.

To further understand the interaction of the parasite J domains with chaperones, we need to envision the whole chaperone-cochaperone complex. A hybrid model of PfHsp70-x_{FL} was created by first generating a homology model with SWISS-MODEL using the recently published full-length ATP-bound DnaK in complex with DnaJ_J structure (PDB 5NRO) as a template [200]. Then, the ATPase domain of the homology model was replaced with the PfHsp70-xATPase:AMP-PNP crystal structure by aligning the region closest to the Hsp40 binding site. The J domain of DnaJ was replaced with either the PFA0660w_J crystal structure or the PFE0055c_J homology model.

Looking at the DnaK_{FL} and DnaJ_J structure, we can see that DnaJ interacts with the ATPase domain of DnaK and the interdomain linker, as had been previously demonstrated, but also the beta-sheet of the substrate binding domain (SBD β). Our hybrid model of PfHsp70-x – PFA0660w_J (Fig. 6.16) reveals that both helices II and III of the J domain would be interacting with PfHsp70-x_{FL} if binding

was similar to DnaK-DnaJ. However, we see that only residues on helix II of PFA0660w_J are affected by PfHsp70-x binding, suggesting that only the interaction with the ATPase domain and linker takes place. Contrarily, the hybrid model of PfHsp70-x – PFE0055c_J indicates that the residues on helix III (E122, R123, K124, F125) affected by PfHsp70-x binding would be located near the SBD β implying that PFE0055c_J interacts primarily with the SBD domain but not the ATPase domain or interdomain linker.

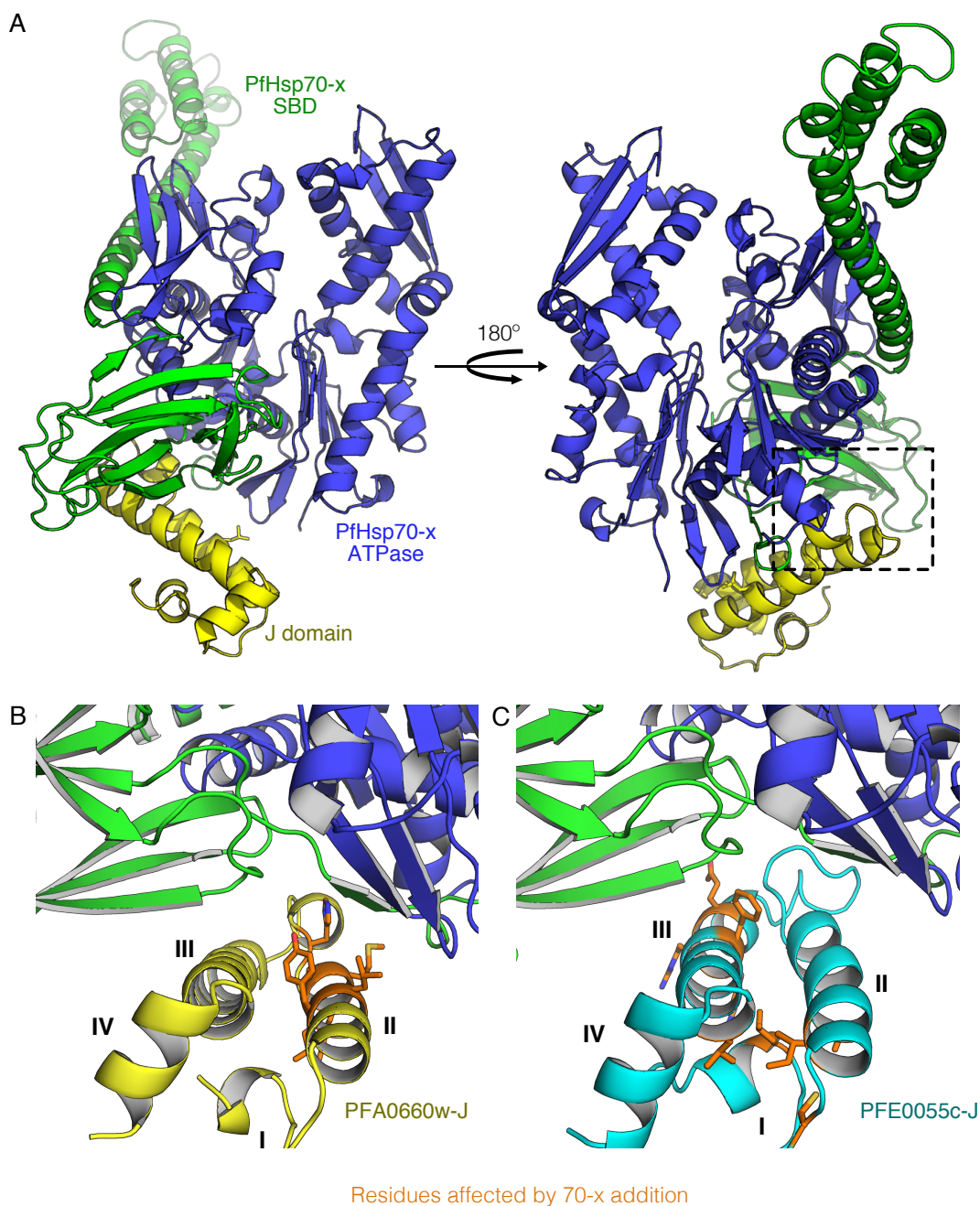


Figure 6.16: Hybrid model of PfHsp70-x and cochaperones. **A:** Cartoon representation of the hybrid model of PfHsp70-x and PFA0660w_J. The hybrid model consists of crystal structures of PfHsp70-x_{ATPase} (blue) and PFA0660w_J (yellow) aligned to a homology model based on the 5NRO structure and the substrate binding domain (green) remaining from the homology model. **B:** A detailed view of the interface of the PfHsp70-x hybrid model with PFA0660w_J (yellow). Residues shown to be significantly affected by PfHsp70-x binding are indicated in orange and shown in stick representation. **C:** A detailed view of the interface of the PfHsp70-x hybrid model with PFE0055c_J (cyan). Residues shown to be significantly affected by PfHsp70-x binding are indicated in orange and shown in stick representation.

6.5 Discussion

In this chapter we aimed to gain insights into the complex that PfHsp70-x forms with cochaperones PFA0660w or PFE0055c. It has been previously shown that the three proteins co-localise in a highly mobile structure named the J-dot within the host erythrocyte cytoplasm, where they may play roles in folding, protecting and trafficking parasite proteins, particularly the virulence factor PfEMP1 [71]. Daniyan *et al* demonstrated that PFA0660w acts as a cochaperone of PfHsp70-x but is not able to significantly stimulate PfHsp70-1 (*P. falciparum* intra-parasitic) or Hsp72 (human heat induced) in a dose-dependent manner [91].

Here, it was found that the specificity of PFA0660w for PfHsp70-x is localised to the J domain despite the high sequence similarity with PFE0055c_J, the promiscuous cochaperone. Studying the pairwise alignment of PFA0660w_J and PFE0055c_J sequences, it was not immediately apparent why PFA0660w is only able to stimulate the activity of PfHsp70-x whereas PFE0055c also stimulates human Hsp70 proteins.

To gain insights into the J domain specificity, we solved the crystal structure of PFA0660w_J. PFA0660w_J consists of 4 α -helices (I - IV) arranged in a similar manner to previously solved structures of other J domains. By mapping amino acid differences, as determined from the pairwise sequence alignment between, PFA0660w and PFE0055c onto the crystal structure of PFA0660w_J, we were able to identify non-conserved regions and potentially important residues such as L103 in PFA0660w. The corresponding residue of L103 in other cochaperones is an arginine or lysine which has been shown to be crucial in the stimulation of Hsp70s. As PFE0055c_J could not be crystallized, a homology model was generated using the crystal structure of PFA0660w_J as a template. The PFE0055c_J homology model was validated using protein energetic conformational analysis and NMR chemical shift restraints.

NMR was then used to determine which residues of PFA0660w_J and PFE0055c_J were involved in binding to PfHsp70-x and Hsc70. A summary of the residues shown to be effected by PfHsp70-x binding is shown in Fig. 6.17. It was clear that the interaction of PFA0660w_J with both chaperones was localised to helix II and that more residues were affected, including L103, when interacting with PfHsp70-x than

with Hsc70 correlating with stimulation levels. In addition, H110 of the PFA0660w_J HPD motif was affected by binding of both Hsc70 and PfHsp70-x unlike D112 which is only affected by Hsc70. PFE0055c_J, however, had a more dispersed pattern of affected residues with effects occurring largely on helix III and a region of buried hydrophobic residues. Additionally, the HPD motif of PFE0055c_J seemed to not be involved in interactions with either PfHsp70-x nor Hsc70.



Figure 6.17: Summary of cochaperone residues affected by PfHsp70-x binding. A pairwise alignment of J domains of PFA0660w and PFE0055c. Residues shown to be involved in PfHsp70-x binding are indicated by triangles (yellow: PFA0660w, cyan: PFE0055c).

A hybrid model based on the recently solved crystal structure of ATP-bound full-length DnaK in complex with DnaJ_J was constructed to understand the affected residues of the J domain in context of the full complex. In the model, residues on the front face of helix II and helix III of the J domain interact with the ATPase domain, interdomain linker and beta sheet of the substrate binding domain of PfHsp70-x. However, we do not see this pattern of residues for either PFA0660w_J nor PFE0055c_J. For PFA0660w_J, we do see helix II residues affected and see that these residues would most likely interact with the ATPase domain and interdomain linker in the hybrid model but there seems to be a lack of interaction with the SBD. We however see the opposite for PFE0055c_J; surface residues affected by PfHsp70-x binding lay largely on helix III which would likely interact with the SBD and we see no residues likely to interact with the ATPase domain of PfHsp70-x.

Residues L103 of PFA0660w and R123 of PFE0055c are attractive candidates for determining specificity as these residues are central in interacting regions and

different between cochaperones. For both residues, we see a change in charge which may lead to differing affinities of the region as different types of interactions are formed. Thus, helix II of *PFA0660w_J* may have an increased affinity for the ATPase domain of *PfHsp70-x* whereas helix III of *PFE0055c_J* has an increased affinity for the SBD. Interestingly, we observed a patch of hydrophobic residues that is formed from the interface of the 4 helices to be affected by *PfHsp70-x* binding. In our model this patch is not found to directly interact with *PfHsp70-x*, however we do still see significant changes in peak intensity in NMR experiments. It is possible that upon binding to *PfHsp70-x*, *PFE0055c* undergoes a slight conformational change that causes the helices to shift in relation to each other and therefore affect the chemical environment of these buried residues. It has previously been reported that J domain helix II of *E. coli* DnaJ bends upon binding to the ATPase domain [201] which supports the idea of slight conformational change of *PFE0055c*.

It is possible that modelling our complex on the basis of DnaK in complex with DnaJ_J may be incorrect, because the DnaK structure was solved in the ATP-bound state whereas we performed our experiments in the absence of nucleotide. The DnaK-DnaJ structure represents an almost-active complex in which the chaperone would be ready to hydrolyse ATP however no client protein is bound to the substrate binding domain. It has been previously shown that the ATP-bound form of Hsp70 docks the substrate binding domain to the ATPase domain forming an interface that the J domain can extensively bind [50, 39]. Perhaps we have characterised a state in which the substrate binding domain is not docked to the ATP domain and so the J domain is not binding at the interface hence why we only see interactions with either the ATPase domain or the SBD - not both. This is likely as the HPD motif is deemed crucial for stimulation of ATPase activity and we do not see this motif engaged in *PFE0055c*.

Further NMR studies must be carried out in the presence of ATP or ATP analogue, AMP-PNP, to determine if we see a change in residues affected by *PfHsp70-x* binding which may closer resemble the DnaK-DnaJ complex. Additional

future work would involve the mutation of residues deemed to be important in specificity such as L103 and E125 of PFA0660w_J.

We have shown that PFA0660w and PFE0055c interact with PfHsp70-x in different ways which may be utilised to design drugs preventing certain Hsp70-Hsp40 complexes from forming and thus fine-tune inhibition of the chaperone network. This work also gives further insights into the complexity of the Hsp70-Hsp40 interaction and specificity determinants which has impact outside the field of malaria and into the field of chaperones, more generally.

7

Discussion

Contents

7.1	Molecular chaperones as antimalarial targets	157
7.2	Druggability of PfHsp70-x_{ATPase}	159
7.3	Targeting the PfHsp70-x/Hsp40 complex	164
7.4	Other exported PfHsp40s	165
7.5	The infected erythrocyte chaperone network	167
7.6	Future work	168
7.7	Conclusions	170

In this thesis, we presented work focusing on PfHsp70-x, the parasite-exported Hsp70, and its J dot resident cochaperones, PFA0660w and PFE0055c. We found that PfHsp70-x plays a role in parasite protection from heat shock in certain life stages of the blood cycle making it a possibly attractive drug target. The crystal structure of the ATPase domain of PfHsp70-x was then solved to probe the possibility of specifically inhibiting the parasite chaperone and to provide information for future rational drug design. Known Hsp70 inhibitors, such as methylene blue, were tested for inhibition of PfHsp70-x's ATP hydrolysis activity. The mechanism of inhibition was further studied by testing inhibitory activity on cochaperone-stimulated ATPase activity and by saturation transfer difference NMR spectroscopy. Certain small molecules displayed differential inhibition of

cochaperone-stimulated PfHsp70-x leading us to explore the interaction of PfHsp70-x with its J dot cochaperone partners – PFA0660w and PFE0055c. The crystal structure of the J domain of PFA0660w, the domain encoding specificity, was solved and used to create a homology model for the PFE0055c J domain. NMR was then used to determine regions on the J domains that interact with PfHsp70-x and found that they differ greatly between the two cochaperones.

Taken together, this information allows us to evaluate PfHsp70-x as a drug target against malaria and provide work towards developing such drugs. The interaction studies of PfHsp70-x with PFA0660w and PFE0055c may also allow the design of specific inhibitors that target these partnerships and do not cross-react with human Hsp70s.

7.1 Molecular chaperones as antimalarial targets

Resistance against widely used antimalarials such as chloroquine and artemisinin has emerged and spread throughout malaria endemic countries [202, 203]. In particular, the spread of resistance against artemisinin and its derivatives [204, 205] is of great concern as the current official recommendation for treatment of *P. falciparum* infections are artemisinin-based combination therapies [206]. Existing antimalarials act on a limited number of biological targets [207] bringing to light the need for therapeutics against novel molecular targets with new treatment strategies to eliminate malaria.

Malarial chaperones make an attractive novel antimalarial target for a number of reasons: a large body of research already exists on the subject of molecular chaperones; molecular chaperones have been implicated in a number of diseases leading to the development of small molecule inhibitors that could be repurposed; certain chaperones are highly conserved suggesting a slower mutation rate which in turn would lead to slower emergence of resistance.

In particular, Hsp70 proteins have been heralded as attractive drug targets as they often lay at the centre of chaperone networks and are responsible for numerous tasks in cellular up-keep and survival. Their critical role in neurodegenerative

diseases and cancer has been demonstrated leading to the development of small molecule modulators. In this thesis, we tested a number of previously developed Hsp70 inhibitors for their inhibition of PfHsp70-x activity towards repurposing them as antimalarials.

Repurposing chaperone inhibitors as antimalarials has recently been explored with some success [208, 99]. In a recent study by Chen *et al*, the Hsp70 inhibitor MKT-077, originally developed as an anti-cancer drug, was found to be particularly toxic to *P. falciparum in vitro* – the drug was 14 times more potent to *P. falciparum* parasites than to human cells. The authors propose this effect partially comes from inhibiting PfHsp70-2, the parasite ER resident Hsp70, but suggest that it acts through other targets too. Here, we have shown that amongst other Hsp70 inhibitors, MKT-077 is capable of inhibiting PfHsp70-x, suggesting PfHsp70-x inhibition contributes to the antiplasmodial activity of the small molecule. Methylene blue, a stress-induced Hsp70 inhibitor tested in this thesis, has been historically used as an antimalarial and there has been renewed interest in the dye as recent clinical trials are proving to be successful [175, 173]. Methylene blue has potent antiplasmodial activity, acting on several stages of the parasite’s life cycle. Here we have shown that methylene blue inhibits PfHsp70-x but were not able to confirm if inhibition functions through the same mechanism as that reported for Hsp72. It is possible that methylene blue’s inhibition of PfHsp70-x and Hsp72 contribute to the antimalarial activity of the drug.

Ideally, only plasmodial Hsp70 proteins would be targeted as inhibiting host Hsp70 proteins could have unwanted effects. PfHsp70-x is an especially attractive drug target as small molecule inhibitors are required to cross fewer membranes compared to targeting an intraparasitic protein. Charnaud *et al*’s [82] findings linking PfHsp70-x to virulence, alongside our work showing PfHsp70-x’s role in proliferation and protection during heat shock, confirm the possibility of PfHsp70-x as a drug target.

Aside from creating specific inhibitors of PfHsp70-x for use as an antimalarial, such small molecules could be used as a tool to further understand the role of

PfHsp70-x in the infected red blood cell. Inhibition of PfHsp70-x by small molecules will allow more advanced cell biology studies to be carried out, for example PfHsp70-x could be inhibited alongside the knockout of another protein.

7.2 Druggability of PfHsp70-x_{ATPase}

The crystal structure of PfHsp70-x_{ATPase}, the catalytic domain of PfHsp70-x, was solved to enable the use of structure-based drug design methods in the future. The ATPase domain was chosen as it is the site of nucleotide binding and hydrolysis which is closely linked to chaperone activity and, thus, if ATP hydrolysis is prevented then the chaperone activity is also inhibited. It was found that the structure of PfHsp70-x_{ATPase} was similar in overall fold to human Hsp72 but several patches of non-conserved residues were identified, despite overall high sequence similarity, that could be utilised for specifically targeting PfHsp70-x to prevent adverse effects.

However, the question of PfHsp70-x_{ATPase} druggability remains. To resolve this open question, we identified druggable pockets in the structure of PfHsp70-x_{ATPase}:AMP-PNP using the PockDrug server [209]. PockDrug defines a pocket as druggable if the averaged probability from the 7 best linear discriminant analysis models exceeds 0.5. A set of 9 physicochemical descriptors are used in these different models to determine druggability probability including frequency of aromatic residues, hydrophobicity, volume, geometry and surface area. Three druggable pockets were identified (Fig. 7.1) with two deep pockets found adjacent to the nucleotide binding site (Pockets I and III). Of the nucleotide binding site adjacent pockets, pocket III is similar to that predicted to be the binding site of MKT-077 [179] whereas pocket I seems to be previously untargeted. Pocket II, a smaller pocket on the protein surface, is found far from the nucleotide binding site close to where dihydropyrimidine inhibitors and cochaperones are predicted to bind [176].

Not only are we interested in the druggability of the ATPase domain of PfHsp70-x, but also in the possibility of specifically targeting PfHsp70-x. To assess whether any of the identified druggable pockets are present in human Hsp70 ATPase domains, we repeated the druggability analysis on human Hsp70 structures (Fig. 7.1). The

PfHsp70-x_{ATPase}:AMP-PNP structure was used to search for the most similar human Hsp70 structures in the PDB using the DALI server [210]. The crystal structures of Hsp72 (PDB 3ATU) [211] and Hsc70 (PDB 4H5T) [212] ATPase domains in complex with ADP were found to be highly similar in structure and considered to be particularly relevant as both Hsp70's are found in the red blood cell cytoplasm. Pockets I and III from PfHsp70-x are also found in both human Hsp70 structures with very similar size and overall shape. Pocket II was not found in either of the human structures, although Hsp72 was predicted to have two druggable pockets on either side of where pocket II would be. Pocket II may therefore be an interesting target for specifically inhibiting PfHsp70-x.

Looking closer at Pocket II, we can see that it is formed between two α -helices and two strands from a β -sheet curved around one of the aforementioned helices (Fig. 7.1D). This β -sheet stretches up into the nucleotide binding site where it may be important for properly coordinating ATP for hydrolysis. Designing a small molecule capable of binding this small pocket may therefore prevent the proper arrangement of residues in the catalytic site and thus inhibit the PfHsp70-x ATPase activity.

Pocket II is in close proximity to where Hsp40s are predicted to bind. On the hybrid model of PfHsp70-x and PFA0660w_J created in Chapter 6, we can see that pocket II is adjacent to where PFA0660w_J is predicted bind (Fig. 7.2). NMR data supports the interaction of PFA0660w_J with the ATPase domain and interdomain linker of PfHsp70-x, whereas PFE0055c_J is predicted to interact more with the SBD. We envisage targeting pocket II with a small molecule that extends to the binding site of PFA0660w_J to selectively disrupt the PfHsp70-x and PFA0660w interaction. The hybrid model also shows us that this pocket is not obstructed by the SBD in the context of the full-length protein.

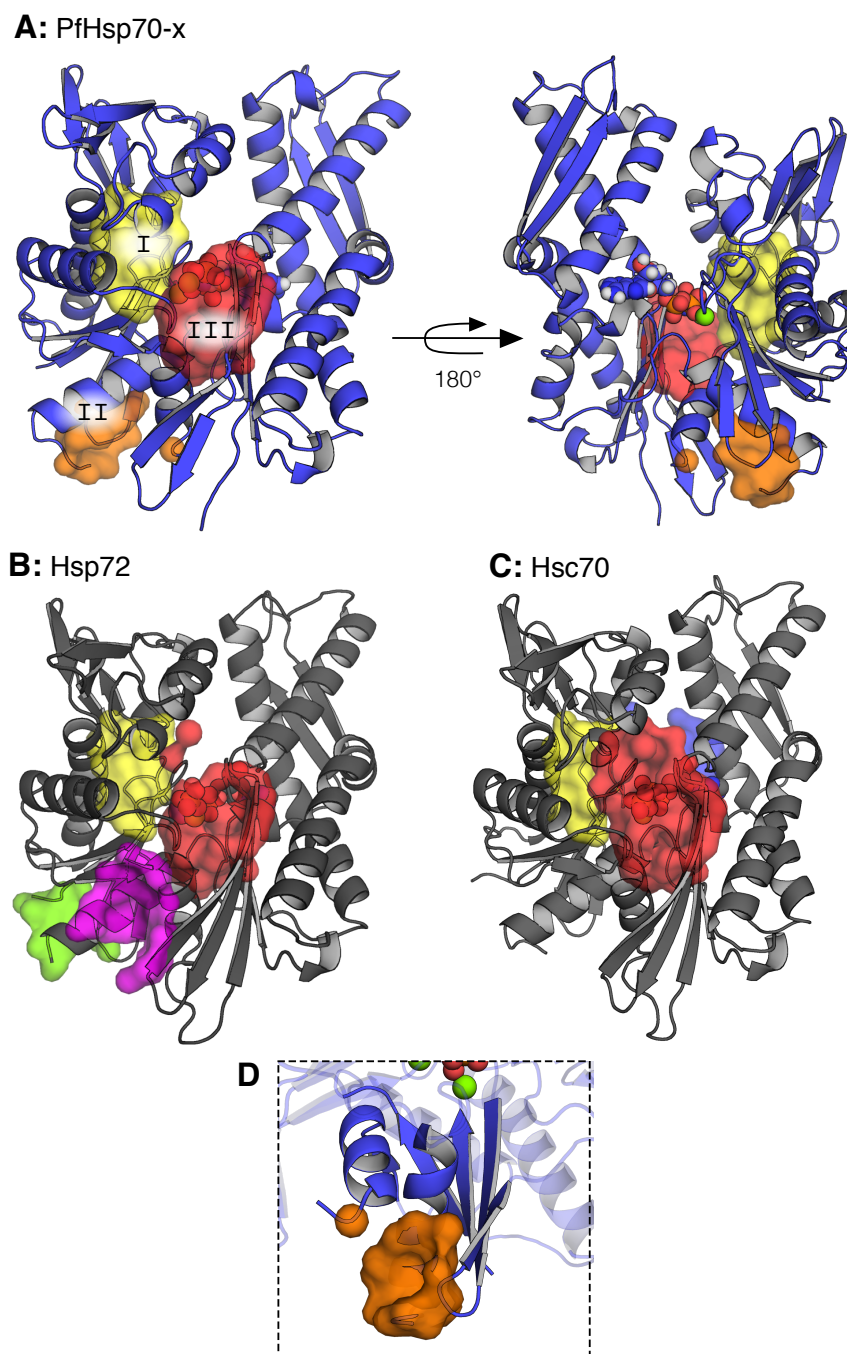


Figure 7.1: Druggable pockets of PfHsp70-x, Hsc70 and Hsp72. **A:** Druggable pockets of the ATPase domain of PfHsp70-x from front and back views. AMP-PNP is shown by spherical representation and pockets are shown by surfaces. **B:** Druggable pockets of the ATPase domain of Hsp72 (PDB 3ATU). ADP is shown by spherical representation and pockets are shown by surfaces. **C:** Druggable pockets of the ATPase domain of Hsc70 (PDB 4H5T). ADP is shown by spherical representation and pockets are shown by surfaces. **D:** A close-up of pocket II, a unique pocket of PfHsp70-x. The pocket consists of two short helices and two strands from a curved beta-sheet on the underside of the ATPase domain. The curved beta-sheet reaches towards the active site.

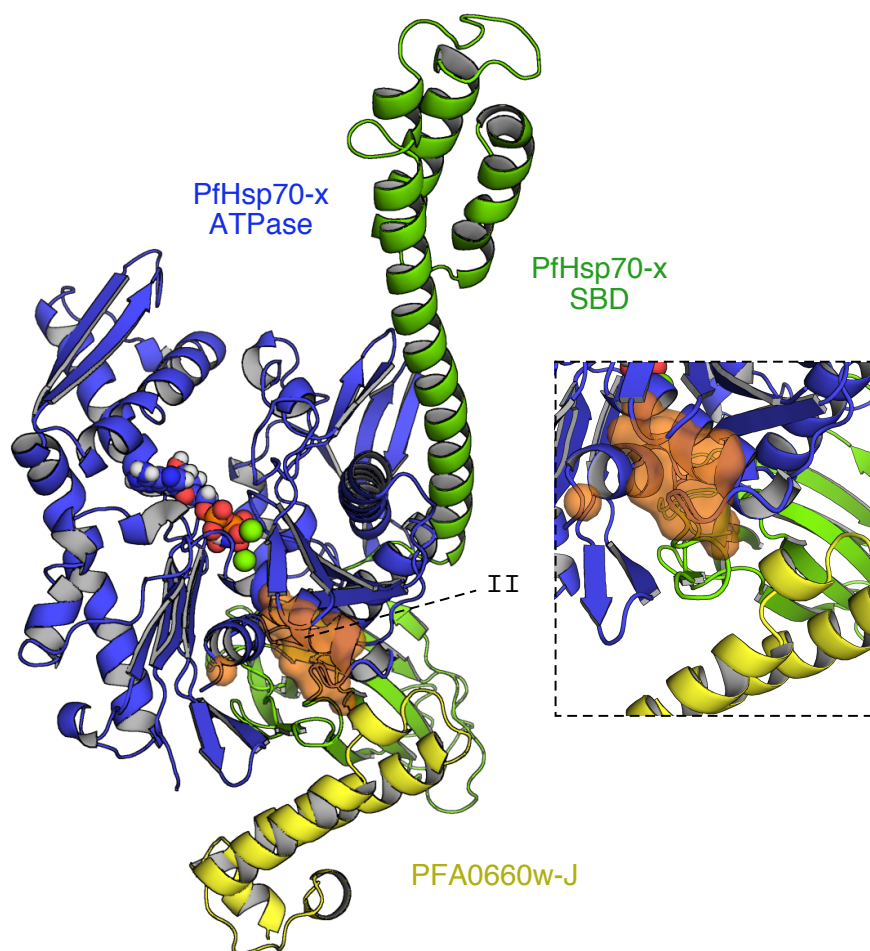


Figure 7.2: Pocket II proximity to J domain binding site. Pocket II is highlighted in orange on the hybrid model of PfHsp70-x and PFA0660w_J created in Chapter 6.

To further explore the possibility of designing specific inhibitors, we studied the conservation of the predicted pockets; although pockets I and III are also found in human Hsp70 structures, perhaps there is enough sequence dissimilarity to allow design of specific inhibitors (Fig. 7.3). A multiple sequence alignment of PfHsp70-x and the two human Hsp70s was performed using MAFFT [181, 180]. The alignment was then used to study the conservation of residues predicted to have their side chains involved in pocket formation. It was found that pocket III is entirely conserved as well as present in all structures, making it the least ideal target for specific inhibition. Pocket I is also found to be highly conserved, with just one amino acid change (I127) compared to Hsc70 (valine) but is identical to Hsp72.

Pocket II, a 'unique' pocket, is also found to be quite highly conserved with just one amino acid change (Q393) compared to the lysines found in both Hsp72 and Hsc70.

Upon closer inspection of Pocket II, we see that the pocket is partially formed by the C-terminus. The human Hsp70 ATPase structures are cut off earlier than our construct and so it is not possible to rule out the existence of this pocket in the human structures or that it would not exist in the full length structure.

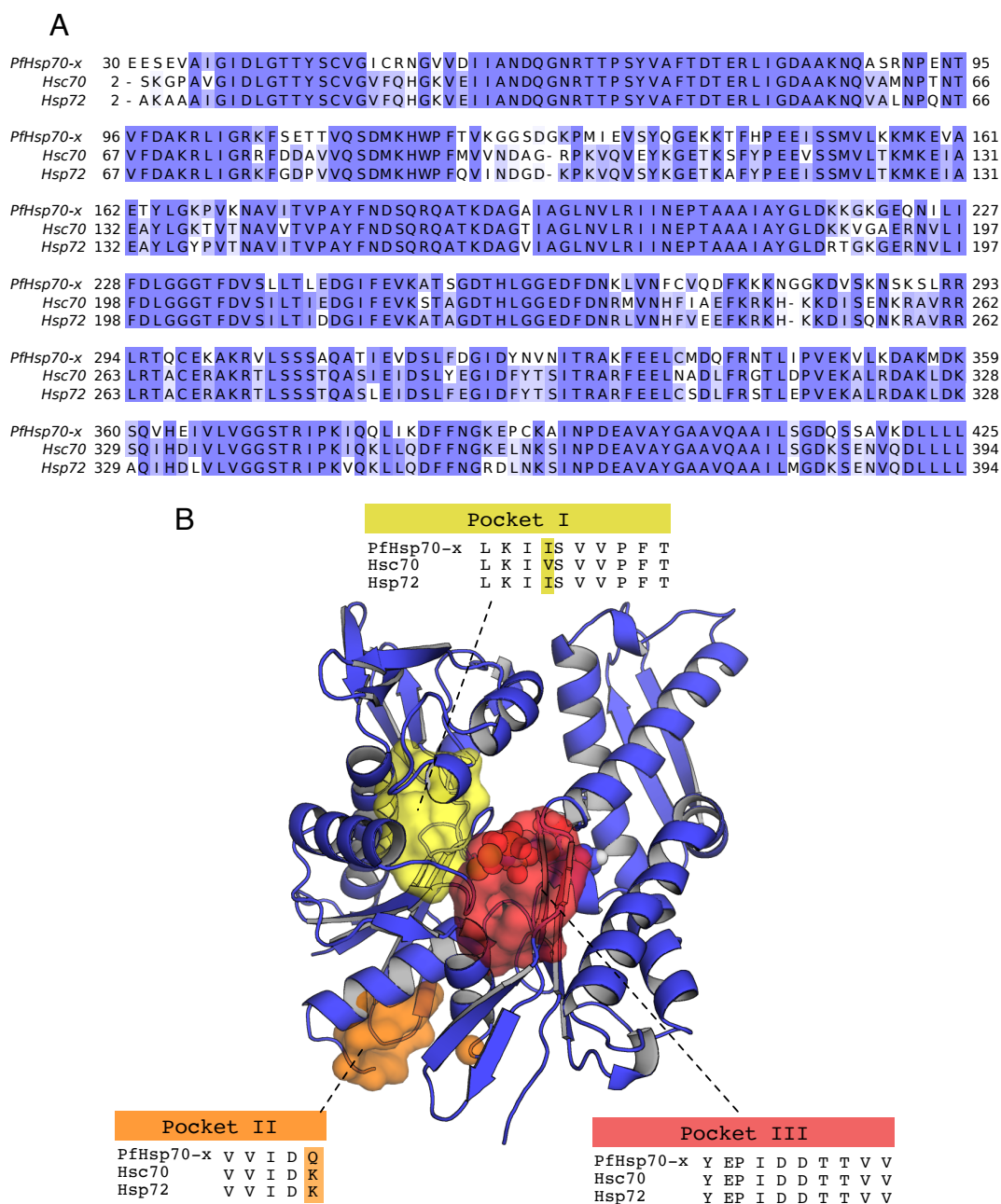


Figure 7.3: Conservation of druggable pockets. **A:** A multiple sequence alignment of ATP domains from PfHsp70-x, Hsc70 and Hsp72 performed by MAFFT [181, 180]. **B:** Sequence alignments of residues predicted to form the pocket with their side chains.

7.3 Targeting the PfHsp70-x/Hsp40 complex

Another approach for targeting PfHsp70-x, is to prevent its interactions with cochaperones that stimulate the otherwise low basal ATP hydrolysis activity. The specificity of Hsp70-Hsp40 interactions have been previously shown [213, 214, 215]

and, in this thesis, we have demonstrated that PFA0660w, a J dot resident parasite Hsp40, preferentially stimulates PfHsp70-x through its J domain. Therefore, the interface of PfHsp70-x and PFA0660w may make an attractive target for small molecules especially as PFA0660w has been suggested to be essential [90].

Until recently, targeting protein-protein interactions has been considered extremely difficult but recent advances and numerous successful examples have changed this perspective [216]. By identifying residues crucial for the interaction, known as ‘hot-spots’, one can design a small molecule that strongly interacts with such hot-spot residues to disrupt protein-protein interactions. In Chapter 6, we identified a number of residues on PFA0660w_J affected by Hsp70 binding in general, whereas other residues, such as L103, are only involved in PfHsp70-x binding. The importance of such unique residues in the interaction with and stimulation of PfHsp70-x needs to be confirmed by mutagenesis; our work provides a starting place for such studies. Small molecules can then be designed to strongly interact with such hot-spot residues of PFA0660w. The PFA0660w residues involved in the PfHsp70-x interaction are highly localised to helix II which makes the design of small molecules particularly easy as they only need to cover a small area. Alternatively, PFE0055c may also make for a good target as it seems to bind PfHsp70-x and Hsp72 in similar ways meaning a single small molecule could prevent PFE0055c from stimulating both.

7.4 Other exported PfHsp40s

This thesis was primarily focused on PfHsp70-x and its interactions with PFA0660w and PFE0055c Hsp40s, as all three proteins were found to colocalise in J dots with PfEMP1 and thus are linked to virulence. However, *P. falciparum* exports many more Hsp40 proteins into the infected red blood cell.

Of the 49 members of the *P. falciparum* Hsp40 family, 18 proteins are predicted to be exported to the red blood cell cytosol (Fig. 7.4) [217, 66, 90]. In addition to PFA0660w and PFE0055c, *P. falciparum* exports one more type II Hsp40, KAHsp40, which has been shown to associate with knobs [218]. Four type III Hsp40 proteins and eleven type IV Hsp40 proteins are exported. A number of the exported Hsp40

proteins also contain a PHIST domain, which is a domain unique to *Plasmodium* and thought to have a range of functions including PfEMP1 binding [69, 219]. For example, the ring-stage erythrocyte surface antigen (RESA) contains both a J domain and a PHIST domain. RESA is technically not exported but is released by the parasite upon invasion and has been implicated in invasion, membrane dynamics and protection during fever in the ring-stage [220, 221]. It is thought that RESA prevents spectrin unfolding and aggregation during fever via its J domain. Another type IV PHIST-containing proteins is mature parasite-infected erythrocyte surface antigen (MESA) which is also implicated in modification of the host cell membrane [222] through binding to cytoskeletal components.

Further work is required to determine if the aforementioned exported PfHs40s are capable of stimulating the activity of PfHsp70-x and/or the host cell chaperones. Those containing both a J domain and PHIST domain, such as RESA, are of particular interest as they may impart specialised functions to chaperones by bringing particular substrates via the PHIST domain.

The knob associated KAHsp40 is also of great interest, as the human Hsp70, Hsc70, is known to relocate to the host cell plasma membrane upon parasite infection [223]. It is possible that KAHsp40 is responsible for the relocation of Hsc70 and that this chaperone-cochaperone complex plays an important role in correct knob formation and PfEMP1 presentation. It has been shown that PFE0055c is capable of stimulating the ATPase activity of human Hsp70s in addition to PfHsp70-x and that a human Hsp40, Hdj1, can stimulate PfHsp70-x and PfHsp70-1 [85, 91]. Thus, one can envisage that KAHsp40 may also be able to interact with human Hsp70 to allow its relocation. In this manner, we start to visualise a complex network of host-parasite Hsp70-Hsp40 interactions within the infected erythrocyte, as host proteins are hijacked and redirected towards pathogenic purposes.

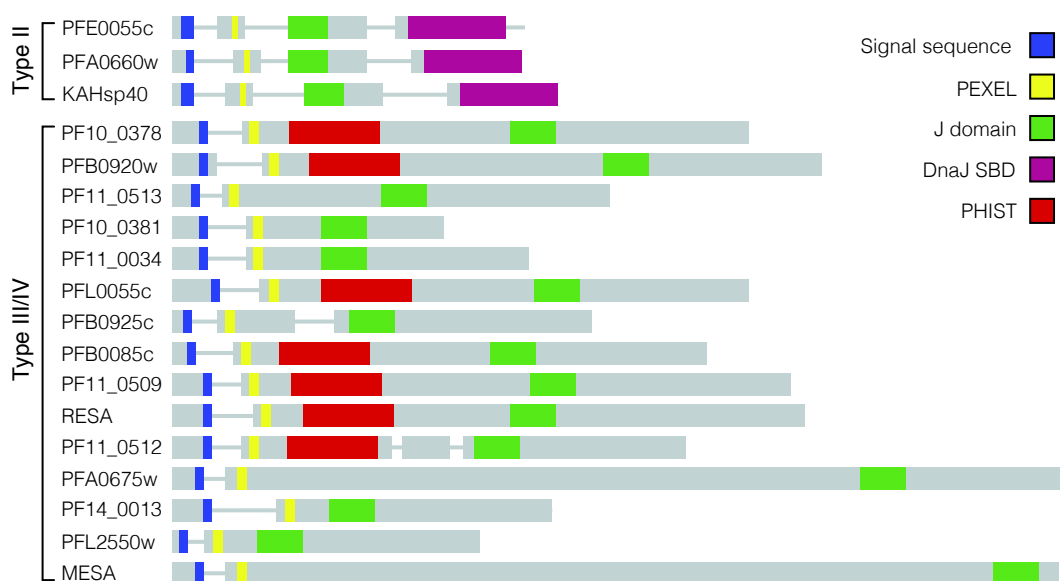


Figure 7.4: Exported Hsp40s of *P. falciparum*. Domain organisation and classification of exported PfHsp40 proteins. A subclass of the Hsp40s contain a PHIST domain that may impart particular functions or allow specific localisation to certain protein complexes. Adapted from [69].

7.5 The infected erythrocyte chaperone network

The picture becomes more complex when we consider the whole chaperone network of the infected erythrocyte (Fig. 7.5). Red blood cells contain several human chaperones including Hsp70s, such as Hsp72 and Hsc70, an Hsp90, the chaperonin TriC, several Hsp40s and small heat shock proteins [224]. Recent work by Mabate *et al* showed that PfHsp70-x interacts with the human Hsp70-Hsp90 organising protein (Hop) suggesting that PfHsp70-x would be capable of working in concert with human Hsp90 in the infected erythrocyte [81]. Substrates are known to be passed from Hsp70s to the chaperonin TriC and small Hsp's for further folding – it is likely that substrates of PfHsp70-x are also passed to these chaperones. The interactions of PfHsp70-x with other members of the network provides further protein-protein interactions that could be targeted to specifically break parts of the network that contribute to virulence or parasite survival. Examples of such interactions include the interface between PfHsp70-x and the human nucleotide exchange factor, the

interaction between human Hsp70s and PfHsp40s that may redirect them to fold pathogenic substrates or the interaction of PfHsp70-x with Hop and thus Hsp90.

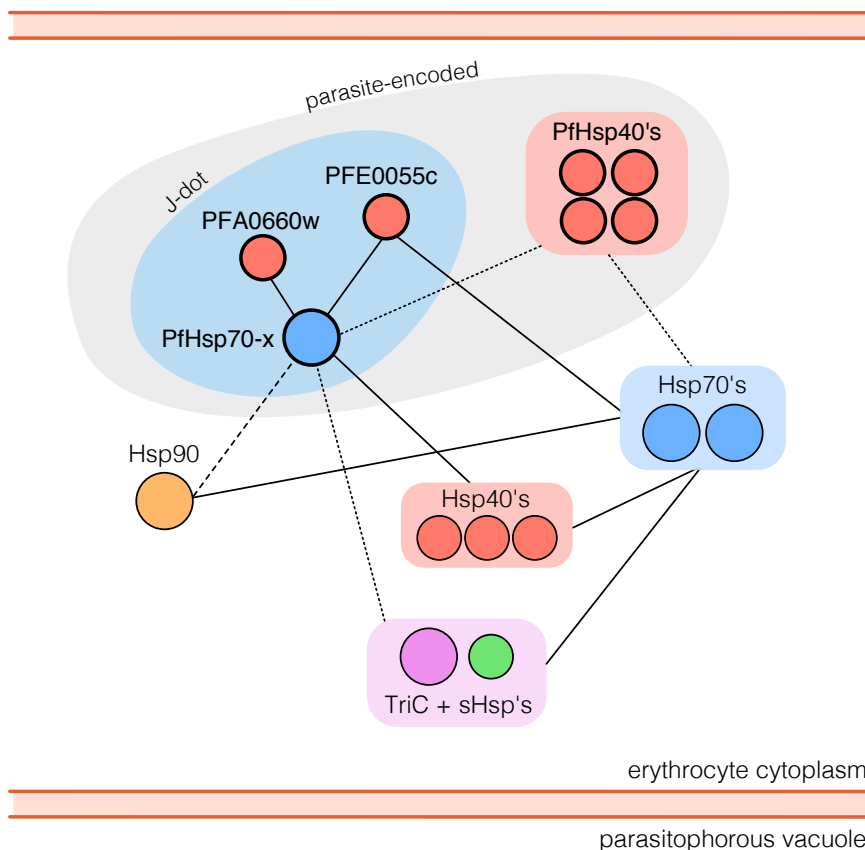


Figure 7.5: The chaperone network of the *P. falciparum* infected erythrocyte. A proposed network of protein interactions between parasite-derived and human chaperones in the cytosol of the infected red blood cell. Confirmed interactions are shown with a solid line and interactions with some evidence shown by dashed lines.

7.6 Future work

In this thesis, we studied just a small section of the chaperone network in the infected erythrocyte. This information not only provides information towards designing antimalarials that target the chaperone system but also gives an insight into how Hsp40 proteins can specifically stimulate certain Hsp70s. However, further work needs to be carried out to confirm such interactions in the red blood cell and to expand our knowledge of the infected erythrocyte chaperone network.

Additional *in vitro* studies would confirm the role of PfHsp70-x and colocalised

Hsp40's in the erythrocyte. For example, measuring expression levels of PfHsp70-x in response to heat shock will confirm the role of PfHsp70-x in protection against stress. It was previously reported that heat shock increases cytoadherence of infected erythrocytes suggesting fever is beneficial to the parasite [80]. Charnaud *et al* demonstrated a link between PfHsp70-x and export of PfEMP1 and so an experiment in which parasites lacking PfHsp70-x were tested for cytoadherence post-heat shock could confirm the role of PfHsp70-x in such an effect of heat shock. Previous attempts to knock out PFA0660w have been unsuccessful suggesting this Hsp40 is essential for parasite viability however this is an assumption and does give further insights to the role of the protein [90]. Thus, creation of a PFA0660w conditional knockdown line would allow further studies and testing for effect on virulence and parasite viability. In addition, a double conditional knockdown of PfHsp70-x and PFA0660w would be useful for predicting the effect of disrupting the PfHsp70-x – PFA0660w interaction with small molecules. We began the creation of PFA0660w knockdown parasite lines for such future studies (Appendix B).

To develop drugs targeting the PfHsp70-x-Hsp40 interaction, further work needs to be carried out to confirm interacting residues and identify hot-spots. NMR titrations of PFA0660w and PFE0055c with PfHsp70-x need to be repeated in the presence of the ATP analogue AMP-PNP to allow full comparison to the published structure of DnaK in complex with DnaJ [35]. Swapping the J domains from PFA0660w and PFE0055c and testing the full-length Hsp40's for specificity of interaction would confirm the localisation of specificity to the J domain alone. Then, mutagenesis of residues deemed important for interaction with PfHsp70-x, such as L103, needs to be performed on full-length Hsp40's and levels of chaperone stimulation measured to confirm hot-spot residues.

Towards the creation of specific inhibitors of PfHsp70-x, structure determination of the substrate binding domain or even the full-length protein may help identify additional druggable pockets unique to PfHsp70-x. Initial crystallisation trials of PfHsp70-x_{SBD} and PfHsp70-x_{FL} were unsuccessful but construct redesign and optimisation may aid crystallisation.

Further work also involves the characterisation of the rest of chaperone network in the erythrocyte. Understanding to what extent the human Hsp70 chaperones are involved in the folding and trafficking of virulence-causing proteins and with which Hsp40s they cooperate with, may change the strategy of targeting the chaperone network. Possible experiments to gain insights into substrate preference include pulldown assays and further knockdowns/knockouts. However, it is difficult to genetically modify a red blood cell to knockout the human Hsp70 and other human chaperones – such modifications would need to be carried out on progenitor cells. Alternative methods may be used such as lysing red blood cells to release their contents before resealing and infecting with the parasite to follow infection without human components of the chaperone network. It is then possible to genetically modify the parasite to export human proteins into the red blood cell to allow complementation studies to study the effects of human chaperones one at a time.

7.7 Conclusions

We have found that PfHsp70-x plays a role in proliferation and survival of the parasite in certain life cycle stages during heat shock. This, taken together with Charnaud's work linking PfHsp70-x to virulence of *P. falciparum*, confirms PfHsp70-x as a drug target. Hsp70 inhibitors could be repurposed as antimalarials providing the compounds have a greater affinity for parasite chaperones than human chaperones. Crystal structures of PfHsp70-x and PFA0660w domains have provided information for future structure-based design. The interaction of PfHsp70-x with J dot resident Hsp40's could also be disrupted by small molecules as residues involved in interactions are concentrated to small areas of the J domain's surface. Further work needs to be carried out to determine the role of human chaperones in virulence and parasite survival and how they are hijacked by parasite proteins possibly through the parasite exported Hsp40's.

Appendices



Construct cloning primers

The constructs described in Chapter 2 Table A.1 were created by amplifying DNA using PCR. For PCR, a number of custom oligonucleotides (Sigma) were designed to amplify specific fragments of the DNA template and to add overhangs for use in Gibson Assembly (New England Biolabs). Provided here is a list of the primers used for DNA amplification in the creation of each construct.

Table A.1: Table of primers.

Construct name	Protein (UniProt)	Forward primer 5' – 3'	Reverse primer 5' – 3'
PfHsp70-x _{FL}	PfHsp70-x (K7N1P5)	GTTCTGTTCCAGGGGCGCGCAGAGGAATCGGAGGTAGC	TTAGCAGCCGGATCTCTTAGTTAACTTC
PfHsp70-x _{ATPass}	PfHsp70-x (K7N1P5)	GTTCTGTTCCAGGGGCGCGCAGAGGAATCGGAGGTAGC	TTAGCAGCCGGATCTCTCATACCGCTGAGCTCTGGTCG
Hsc70 _{FL}	Hsc70 (P11142)	GTTCTGTTCCAGGGGCGCGCATGTCCAGGGACCTGCAGTTG	GTTAGCAGCCGGATCTCTTTAATCAACCTCTTCAATGGTGGGC
PF-A0660w _J	PF-A0660w (Q812E1)	GTTCTGTTCCAGGGGCGCGCATGGACTATTACACGCTGCTGGG	GTTAGCAGCCGGATCTCTTTATAAGTGTACTTGACACGTT TTTCATTATCAGATAATACC
PFE0055c _J	PFE0055c (Q81489)	GTTCTGTTCCAGGGGCGCGCATGGATGAGACTGACTACTACGCAG	TTAGCAGCCGGATCTCTTTACAGACGAAGAAGCGTAAGAAC TATGAC
DnaJ	DnaJ (P08622)	GTTCTGTTCCAGGGGCGCGCATGGCTAAGCAAGATTATTATT ACGAGATTTTAG	GTTAGCAGCCGGATCTCTCAGCGGGTCAAGGTCGTC

B

Generation of PFA0660w knockdown parasite lines

Towards furthering our understanding of PFA0660w's role in the infected red blood cell, we aimed to generate new parasite lines with a destabilisation domain (DD) tag on PFA0660w to allow the knockdown of the cochaperone. The DD tag causes the protein to be targeted for degradation unless in the presence of Sheild-1, a small molecule capable of stabilising the domain and preventing degradation. Thus, the tagged protein can be conditionally knockdown by removing Sheild-1 from culture.

Previous attempts to knockout PFA0660w have been unsuccessful [90] implying that it is essential for parasite viability but this gives us no insight into the exact function of the Hsp40. Additionally, the inability to knockout a protein relating to essentiality is an assumption that may not always be correct.

B.1 Construct cloning

To add the DD tag to the 3' end of PFA0660w we employed CRISPR/Cas9 technology to directly modify the genome of *P. falciparum*. We used a single-plasmid system, pCRISPR-HADD2A (Fig. B.1A), which contains both the CRISPR-associated endonuclease 9 (Cas9) coding sequence and guide RNA (gRNA) necessary for CRISPR/Cas9 genetic modification and was provided by the Beck lab and designed

by Dr Beatrice Schibler. The plasmid also contained blasticidin S and ampicillin resistance genes, BSD and bla respectively, for selection purposes.

The tag to be introduced to the 3' end of the PFA0660w gene contained a 3x hemagglutinin tag (3xHA) and ddFKBP destabilisation domain (DD) linked to the BSD gene by a 2A self-cleaving peptide linker. Homology regions (HR) flank the tag to allow proper insertion to the genome (Fig. B.1B). The 3'HR was amplified from 3D7 genomic DNA using PCR and the 5'HR with mutated protospacer adjacent motif (PAM) to prevent re-snipping was ordered from Genscript. HR's were inserted into the plasmid using In-Fusion (Clontech) or by restriction enzyme digestion and ligation (New England Biolabs).

Oligonucleotides (Sigma) were purchased and annealed to create the PFA0660w double-stranded gRNA (Fig. B.1C) to complete the gRNA necessary for guiding Cas9 to the correct location. The double-stranded gRNA was inserted into pCRISPR-HADD2A using restriction enzyme digestion and ligation.

E. coli DH5 α cells were used for plasmid multiplication and a Qiagen MaxiPrep kit was used to extract and purify the plasmid DNA according to manufacturer's instructions.

The sequence of all primers used for DNA amplification and gRNA creation for pCRISPR-HADD2A-PFA0660w can be found in Table B.1.

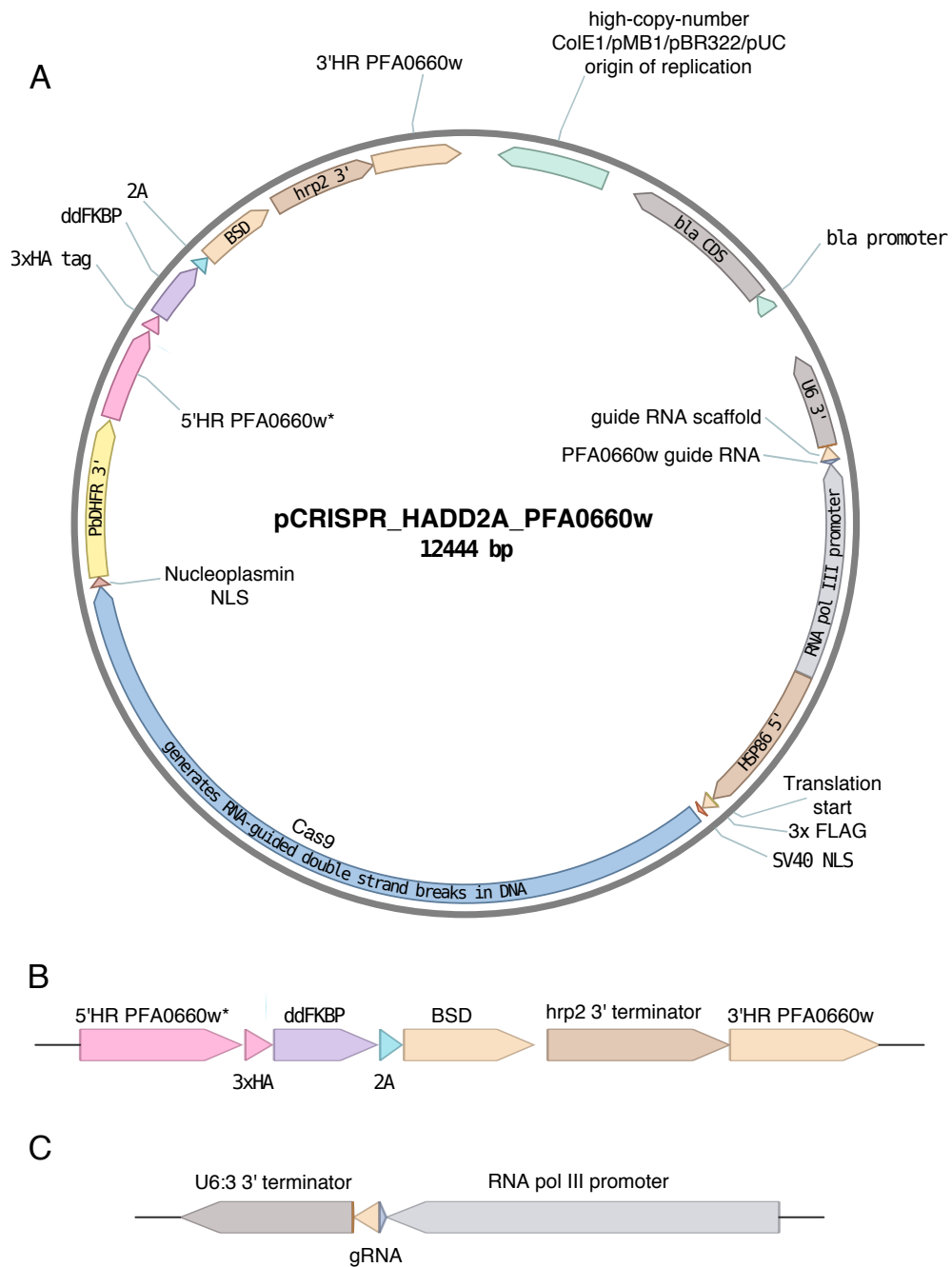


Figure B.1: The pCRISPR-HADD2A-PFA0660w plasmid. Overlaid spectra of PFA0660w_J (red) and PFA0660w_J with either PfHsp70-x or Hsc70 (green).

B.2 Transfection

Highly synchronised *P. falciparum* 3D7 cultures containing >5 % young ring-stage parasites were centrifuged at 600 x g and medium removed. 100 µg of plasmid was

Table B.1: Table of primers CRISPR-HADD2A-PFA0660w.

Primer name	DNA fragment	Primer sequence 5' – 3'
5' HR forward	5'HR	CAGGGTAGCTGATATCGGATCCGAAGA TTTATACAACGGGTGCACAAAAACA TTAAAAAG
5' HR reverse	5'HR	CGTACGGGTACTTAAGCCGCGGATAAC TCTCTTTTAAATA TCTCTTTT TCGAA TTTT TTTTGCTC
3' HR forward	3'HR	CATATTTTAAATCTAGAA TTOGAGATA TTTAAAGAGAGTTAT TAAAAAATAAAAA CACA TAC
3' HR reverse	3'HR	CTTGTCGACGGAGCTCGCTAGCAC TTTGTTT TTA TTACATCAAAATATGAATATCTAAAAACC
gRNA forward (Bsal)	gRNA	AAACTTTTATTGTATTAGGATA T
gRNA reverse (Bsal)	gRNA	TATTATATCCTAATACAA TAAAAA

mixed with cytomix (25 mM HEPES, 10 mM K₂HPO₄, 120 mM KCl, 0.15 mM CaCl₂, 2 mM EGTA, 5 mM MgCl₂ pH 7.6). The cell pellet was resuspended in DNA-cytomix mixture. Cells were electroporated using either a double square or a single exponential pulse: double square consisting of two 4 ms pulses of 310 V with a 2 second interval; single exponential waveform with voltage of 310 V, 950 μ F capacitance and infinite resistance. Electroporated cells were added to fresh culturing medium and blood. After 8 hours, 2.5 mg mL⁻¹ blasticidin and 625 nM Shield-1 were added to cultures. Fresh blood was added to cultures 24 hours post electroporation. Culture medium was changed every 24 hours for one week and then every 48 hours from then on with blood added as required. Samples were taken for Giemsa staining every 48 hours to check for the presence of parasites.

After the appearance of many ring-stage parasites, the parasites were subcultured to create emergency freezings and for future culturing. Sorbitol synchronisation was used to enrich for ring-stage parasites. Cultures containing 10 % young rings were centrifuged at 600 x g for 5 minutes and the culturing medium removed. Freezing solution (28 % Glycerol, 3 % Sorbitol, 0.65 % NaCl) was gently added to the cell pellet then the mixture transferred to a cryovial before being flash frozen in liquid nitrogen.

B.3 Current status

Of all the transfected cultures that regrew after addition of blasticidin for selection, only one line had successfully incorporated the HA-DD tag. This was determined through PCR and Western blot. Unfortunately, the only line that was successfully modified did not also carry a knockdown tag for PfHsp70-x and so we did not create a double knockdown parasite. However, the generation of a PFA0660w conditional knockdown line will be useful for future studies. The addition of the HA tag alongside the DD domain will enable localisation studies through and anti-HA antibody as currently there is no anti-PFA0660w antibody.

References

- [1] World Health Organization. Global Malaria Programme. *World malaria report 2017*.
- [2] Christopher JL Murray et al. “Global malaria mortality between 1980 and 2010: a systematic analysis”. In: *The Lancet* 379.9814 (2012), pp. 413–431.
- [3] E.Y. Klein. “Antimalarial drug resistance: a review of the biology and strategies to delay emergence and spread”. In: *International Journal of Antimicrobial Agents* 41.4 (2013), pp. 311–317.
- [4] Andrej Trampuz et al. “Clinical review: Severe malaria.” In: *Critical care (London, England)* 7.4 (2003), pp. 315–23.
- [5] Louis H. Miller et al. “The pathogenic basis of malaria”. In: *Nature* 415.6872 (2002), pp. 673–679.
- [6] Alan F. Cowman et al. *Malaria: Biology and Disease*. 2016.
- [7] Gabrielle A. Josling, Kim C. Williamson, and Manuel Llinás. “Regulation of Sexual Commitment and Gametocytogenesis in Malaria Parasites”. In: *Annual Review of Microbiology* 72.1 (2018), pp. 501–519.
- [8] K Lingelbach, KA Joiner - Journal of cell Science, and undefined 1998. “The parasitophorous vacuole membrane surrounding Plasmodium and Toxoplasma: an unusual compartment in infected cells”. In: *jcs.biologists.org* ().
- [9] G E Ward et al. “The origin of parasitophorous vacuole membrane lipids in malaria-infected erythrocytes.” In: *Journal of cell science* 106 (Pt 1.1 (1993), pp. 237–48.
- [10] Tobias Spielmann et al. “Molecular make-up of the Plasmodium parasitophorous vacuolar membrane”. In: *International Journal of Medical Microbiology* 302.4-5 (2012), pp. 179–186.
- [11] Louise E. Kemp, Masahiro Yamamoto, and Dominique Soldati-Favre. “Subversion of host cellular functions by the apicomplexan parasites”. In: *FEMS Microbiology Reviews* 37.4 (2013), pp. 607–631.
- [12] Melissa Khosh-Naucke et al. “Identification of novel parasitophorous vacuole proteins in *P. falciparum* parasites using BioID”. In: *International Journal of Medical Microbiology* 308.1 (2018), pp. 13–24.
- [13] Marcel Deponste et al. “Wherever I may roam: Protein and membrane trafficking in *P. falciparum*-infected red blood cells”. In: *Molecular and Biochemical Parasitology* 186.2 (2012), pp. 95–116.
- [14] Tania F. de Koning-Ward et al. “A newly discovered protein export machine in malaria parasites”. In: *Nature* 459.7249 (2009), pp. 945–949.

- [15] I Ansorge et al. "Protein sorting in *Plasmodium falciparum*-infected red blood cells permeabilized with the pore-forming protein streptolysin O." In: *The Biochemical journal* 315 (Pt 1.1 (1996), pp. 307–14.
- [16] Nina Gehde et al. "Protein unfolding is an essential requirement for transport across the parasitophorous vacuolar membrane of *Plasmodium falciparum*". In: *Molecular Microbiology* 71.3 (2009), pp. 613–628.
- [17] Sarah C. Charnaud et al. "Spatial organization of protein export in malaria parasite blood stages". In: *Traffic* 19.8 (2018), pp. 605–623.
- [18] Alister G Craig, Mohd Fadzli Mustaffa Khairul, and Pradeep R Patil. "Cytoadherence and severe malaria." In: *The Malaysian journal of medical sciences : MJMS* 19.2 (2012), pp. 5–18.
- [19] Lorena M. Coronado, Christopher T. Nadovich, and Carmenza Spadafora. "Malarial hemozoin: From target to tool". In: *Biochimica et Biophysica Acta (BBA) - General Subjects* 1840.6 (2014), pp. 2032–2041.
- [20] Yuhui Yang et al. "Reactive Oxygen Species in the Immune System". In: *International Reviews of Immunology* 32.3 (2013), pp. 249–270.
- [21] F. Ulrich Hartl, Andreas Bracher, and Manajit Hayer-Hartl. "Molecular chaperones in protein folding and proteostasis". In: *Nature* 475.7356 (2011), pp. 324–332.
- [22] Bernd Bukau et al. "Molecular chaperones and protein quality control." In: *Cell* 125.3 (2006), pp. 443–51.
- [23] F. Ulrich Hartl. "Protein Misfolding Diseases". In: *Annual Review of Biochemistry* 86.1 (2017), pp. 21–26.
- [24] F. Ritossa. "A new puffing pattern induced by temperature shock and DNP in drosophila". In: *Experientia* 18.12 (1962), pp. 571–573.
- [25] F Ritossa. "Discovery of the heat shock response." In: *Cell stress & chaperones* 1.2 (1996), pp. 97–8.
- [26] M J Schlesinger. "Heat shock proteins." In: *The Journal of biological chemistry* 265.21 (1990), pp. 12111–4.
- [27] Harm H. Kampinga et al. "Guidelines for the nomenclature of the human heat shock proteins". In: *Cell Stress and Chaperones* 14.1 (2009), pp. 105–111.
- [28] W R Boorstein, T Ziegelhoffer, and E A Craig. "Molecular evolution of the HSP70 multigene family." In: *Journal of molecular evolution* 38.1 (1994), pp. 1–17.
- [29] Eirini Meimaridou, Sakina B Gooljar, and J Paul Chapple. "From hatching to dispatching: the multiple cellular roles of the Hsp70 molecular chaperone machinery". In: *Journal of Molecular Endocrinology* 42.1 (2009), pp. 1–9.
- [30] Mehdi Kabani and Céline N Martineau. "Multiple hsp70 isoforms in the eukaryotic cytosol: mere redundancy or functional specificity?" In: *Current genomics* 9.5 (2008), pp. 338–248.
- [31] C G Evans, L Chang, and J E Gestwicki. "Heat shock protein 70 (hsp70) as an emerging drug target". In: *J Med Chem* 53.12 (2010), pp. 4585–4602.

- [32] Markus Vogel, Matthias P Mayer, and Bernd Bukau. "Allosteric regulation of Hsp70 chaperones involves a conserved interdomain linker." In: *The Journal of biological chemistry* 281.50 (2006), pp. 38705–11.
- [33] K M Flaherty, C DeLuca-Flaherty, and D B McKay. "Three-dimensional structure of the ATPase fragment of a 70K heat-shock cognate protein." In: *Nature* 346.6285 (1990), pp. 623–8.
- [34] S Rüdiger et al. "Substrate specificity of the DnaK chaperone determined by screening cellulose-bound peptide libraries." In: *The EMBO journal* 16.7 (1997), pp. 1501–7.
- [35] Roman Kityk, Jürgen Kopp, and Matthias P. Mayer. "Molecular Mechanism of J-Domain-Triggered ATP Hydrolysis by Hsp70 Chaperones". In: *Molecular Cell* 69.2 (2018), 227–237.e4.
- [36] P. Czihal et al. "Api88 is a novel antibacterial designer Peptide to treat systemic infections with multidrug-resistant gram-negative pathogens." In: *Acs Chem.Biol.* 7 (2012), pp. 1281–1291.
- [37] E B Bertelsen et al. "Solution conformation of wild-type E. coli Hsp70 (DnaK) chaperone complexed with ADP and substrate". eng. In: *Proc Natl Acad Sci U S A* 106.21 (2009), pp. 8471–8476.
- [38] Ruifeng Qi et al. "Allosteric opening of the polypeptide-binding site when an Hsp70 binds ATP". In: *Nature Structural & Molecular Biology* 20.7 (2013), pp. 900–907.
- [39] Joanna F. Swain et al. "Hsp70 Chaperone Ligands Control Domain Association via an Allosteric Mechanism Mediated by the Interdomain Linker". In: *Molecular Cell* 26.1 (2007), pp. 27–39.
- [40] S M Wilbanks et al. "Solution small-angle X-ray scattering study of the molecular chaperone Hsc70 and its subfragments." In: *Biochemistry* 34.38 (1995), pp. 12095–106.
- [41] Yongbo Zhang and Erik R P Zuiderweg. "The 70-kDa heat shock protein chaperone nucleotide-binding domain in solution unveiled as a molecular machine that can reorient its functional subdomains." In: *Proceedings of the National Academy of Sciences of the United States of America* 101.28 (2004), pp. 10272–7.
- [42] M P Mayer and B Bukau. "Hsp70 chaperones: cellular functions and molecular mechanism." In: *Cellular and molecular life sciences : CMLS* 62.6 (2005), pp. 670–84.
- [43] Matthias P Mayer. "Gymnastics of molecular chaperones." In: *Molecular cell* 39.3 (2010), pp. 321–31.
- [44] Roman Kityk et al. "Structure and Dynamics of the ATP-Bound Open Conformation of Hsp70 Chaperones". In: *Molecular Cell* 48.6 (2012), pp. 863–874.
- [45] Bernd Bukau et al. "Multistep mechanism of substrate binding determines chaperone activity of Hsp70." In: *Nature Structural Biology* 7.7 (2000), pp. 586–593.
- [46] A W Karzai and R McMacken. "A bipartite signaling mechanism involved in DnaJ-mediated activation of the Escherichia coli DnaK protein." In: *The Journal of biological chemistry* 271.19 (1996), pp. 11236–46.

- [47] T Laufen et al. “Mechanism of regulation of hsp70 chaperones by DnaJ cochaperones.” In: *Proceedings of the National Academy of Sciences of the United States of America* 96.10 (1999), pp. 5452–7.
- [48] Unekwu M. Yakubu and Kevin A. Morano. “Roles of the nucleotide exchange factor and chaperone Hsp110 in cellular proteostasis and diseases of protein misfolding”. In: *Biological Chemistry* 399.10 (2018), pp. 1215–1221.
- [49] Harm H. Kampinga and Elizabeth A. Craig. “The HSP70 chaperone machinery: J proteins as drivers of functional specificity”. In: *Nat Rev Mol Cell Biol* 11.8 (2010), pp. 579–592.
- [50] R Kityk et al. “Structure and dynamics of the ATP-bound open conformation of Hsp70 chaperones”. eng. In: *Mol Cell* 48.6 (2012), pp. 863–874.
- [51] Matthias P. Mayer. “Hsp70 chaperone dynamics and molecular mechanism”. In: *Trends in Biochemical Sciences* 38.10 (2013), pp. 507–514.
- [52] E A Craig et al. “The diverse roles of J-proteins, the obligate Hsp70 co-chaperone.” In: *Reviews of physiology, biochemistry and pharmacology* 156 (2006), pp. 1–21.
- [53] Jurre Hageman and Harm H. Kampinga. “Computational analysis of the human HSPH/HSPA/DNAJ family and cloning of a human HSPH/HSPA/DNAJ expression library”. In: *Cell Stress and Chaperones* 14.1 (2009), pp. 1–21.
- [54] Douglas M. Cyr and Carlos H. Ramos. “Specification of Hsp70 Function by Type I and Type II Hsp40”. In: Springer, Cham, 2015, pp. 91–102.
- [55] M E Cheetham and A J Caplan. “Structure, function and evolution of DnaJ: conservation and adaptation of chaperone function.” In: *Cell stress & chaperones* 3.1 (1998), pp. 28–36.
- [56] K Ohtsuka and M Hata. “Mammalian HSP40/DNAJ homologs: cloning of novel cDNAs and a proposal for their classification and nomenclature.” In: *Cell stress & chaperones* 5.2 (2000), pp. 98–112.
- [57] Fritha Hennessy et al. “Not all J domains are created equal: implications for the specificity of Hsp40-Hsp70 interactions.” In: *Protein science : a publication of the Protein Society* 14.7 (2005), pp. 1697–709.
- [58] L Goffin and C Georgopoulos. “Genetic and biochemical characterization of mutations affecting the carboxy-terminal domain of the Escherichia coli molecular chaperone DnaJ.” In: *Molecular microbiology* 30.2 (1998), pp. 329–40.
- [59] Z Lu and D M Cyr. “The conserved carboxyl terminus and zinc finger-like domain of the co-chaperone Ydj1 assist Hsp70 in protein folding.” In: *The Journal of biological chemistry* 273.10 (1998), pp. 5970–8.
- [60] Jingzhi Li, Xinguo Qian, and Bingdong Sha. “The crystal structure of the yeast Hsp40 Ydj1 complexed with its peptide substrate.” In: *Structure (London, England : 1993)* 11.12 (2003), pp. 1475–83.
- [61] Thomas R. M. Barends et al. “Combining crystallography and EPR: crystal and solution structures of the multidomain cochaperone DnaJ”. In: *Acta Crystallographica Section D Biological Crystallography* 69.8 (2013), pp. 1540–1552.

- [62] Yuan-yuan Shi, Xin-guo Hong, and Chih-chen Wang. “The C-terminal (331-376) sequence of *Escherichia coli* DnaJ is essential for dimerization and chaperone activity: a small angle X-ray scattering study in solution.” In: *The Journal of biological chemistry* 280.24 (2005), pp. 22761–8.
- [63] M Pellecchia et al. “NMR structure of the J-domain and the Gly/Phe-rich region of the *Escherichia coli* DnaJ chaperone.” In: *Journal of molecular biology* 260.2 (1996), pp. 236–50.
- [64] M. K. Greene, K. Maskos, and S. J. Landry. “Role of the J-domain in the cooperation of Hsp40 with Hsp70”. In: *Proceedings of the National Academy of Sciences* 95.11 (1998), pp. 6108–6113.
- [65] Jin Hae Kim et al. “Nucleotide-Dependent Interactions within a Specialized Hsp70/Hsp40 Complex Involved in Fe–S Cluster Biogenesis”. In: *Journal of the American Chemical Society* 136.33 (2014), pp. 11586–11589.
- [66] Pragyan Acharya, Ranjit Kumar, and Utpal Tatu. “Chaperoning a cellular upheaval in malaria: Heat shock proteins in *Plasmodium falciparum*”. In: *Molecular and Biochemical Parasitology* 153.2 (2007), pp. 85–94.
- [67] Addmore Shonhai, Aileen Boshoff, and Gregory L. Blatch. “The structural and functional diversity of Hsp70 proteins from *Plasmodium falciparum*”. In: *Protein Science* 16.9 (2007), pp. 1803–1818.
- [68] Swati Patankar et al. “Serial Analysis of Gene Expression in *Plasmodium falciparum* Reveals the Global Expression Profile of Erythrocytic Stages and the Presence of Anti-Sense Transcripts in the Malarial Parasite”. In: *Molecular Biology of the Cell* 12.10 (2001). Ed. by David Botstein, pp. 3114–3125.
- [69] T J Sargeant et al. “Lineage-specific expansion of proteins exported to erythrocytes in malaria parasites”. In: *Genome Biol* 7.2 (2006), R12.
- [70] Agnieszka Chacinska et al. “Mitochondrial Presequence Translocase: Switching between TOM Tethering and Motor Recruitment Involves Tim21 and Tim17”. In: *Cell* 120.6 (2005), pp. 817–829.
- [71] S Kulzer et al. “*Plasmodium falciparum*-encoded exported hsp70/hsp40 chaperone/co-chaperone complexes within the host erythrocyte”. In: *Cell Microbiol* 14.11 (2012), pp. 1784–1795.
- [72] J Schulze et al. “The *Plasmodium falciparum* exportome contains non-canonical PEXEL/HT proteins”. In: *Mol Microbiol* (2015).
- [73] M Marti et al. “Signal-mediated export of proteins from the malaria parasite to the host erythrocyte”. In: *J Cell Biol* 171.4 (2005), pp. 587–592.
- [74] D E Goldberg and A F Cowman. “Moving in and renovating: exporting proteins from *Plasmodium* into host erythrocytes”. In: *Nat Rev Microbiol* 8.9 (2010), pp. 617–621.
- [75] L G Pologe and J V Ravetch. “Large deletions result from breakage and healing of *P. falciparum* chromosomes.” In: *Cell* 55.5 (1988), pp. 869–74.
- [76] Dionicia Gamboa et al. “A Large Proportion of *P. falciparum* Isolates in the Amazon Region of Peru Lack pfhrp2 and pfhrp3: Implications for Malaria Rapid Diagnostic Tests”. In: *PLoS ONE* 5.1 (2010). Ed. by Anders Bjorkman, e8091.

- [77] T F de Koning-Ward, P R Gilson, and B S Crabb. “Advances in molecular genetic systems in malaria”. In: *Nat Rev Microbiol* 13.6 (2015), pp. 373–387.
- [78] B Elsworth, B S Crabb, and P R Gilson. “Protein export in malaria parasites: an update”. In: *Cell Microbiol* 16.3 (2014), pp. 355–363.
- [79] Soundara Raghavan Pavithra et al. “Recurrent Fever Promotes Plasmodium falciparum Development in Human Erythrocytes”. In: *Journal of Biological Chemistry* 279.45 (2004), pp. 46692–46699.
- [80] R. Udomsangpetch et al. “Febrile temperatures induce cytoadherence of ring-stage Plasmodium falciparum-infected erythrocytes”. In: *Proceedings of the National Academy of Sciences* 99.18 (2002), pp. 11825–11829.
- [81] Blessing Mabate et al. “Structural and biochemical characterization of Plasmodium falciparum Hsp70-x reveals functional versatility of its C-terminal EEVN motif”. In: *Proteins: Structure, Function, and Bioinformatics* (2018).
- [82] Sarah C. Charnaud et al. “The exported chaperone Hsp70-x supports virulence functions for Plasmodium falciparum blood stage parasites”. In: *PLOS ONE* 12.7 (2017). Ed. by Tobias Spielmann, e0181656.
- [83] David W. Cobb et al. “The Exported Chaperone PfHsp70x Is Dispensable for the Plasmodium falciparum Intraerythrocytic Life Cycle”. In: *mSphere* 2.5 (2017). Ed. by Ira J Blader, e00363–17.
- [84] Ingrid L Cockburn et al. “Screening for small molecule modulators of Hsp70 chaperone activity using protein aggregation suppression assays: inhibition of the plasmodial chaperone PfHsp70-1”. In: *Biol. Chem* 392 (2011), pp. 431–438.
- [85] Ingrid L. Cockburn et al. “Selective modulation of plasmodial Hsp70s by small molecules with antimalarial activity”. In: *Biological Chemistry* 395.11 (2014), pp. 1353–1362.
- [86] Victoria A Assimon et al. “Hsp70 protein complexes as drug targets.” In: *Current pharmaceutical design* 19.3 (2013), pp. 404–17.
- [87] Qi Zhang et al. “Proteomic analysis of exported chaperone/co-chaperone complexes of P. falciparum reveals an array of complex protein-protein interactions.” In: *Scientific reports* 7 (2017), p. 42188.
- [88] S Kulzer et al. “Parasite-encoded Hsp40 proteins define novel mobile structures in the cytosol of the P. falciparum-infected erythrocyte”. In: *Cell Microbiol* 12.10 (2010), pp. 1398–1420.
- [89] Ankita Behl et al. “Cholesterol bound Plasmodium falciparum co-chaperone ‘PFA0660w’ complexes with major virulence factor ‘PfEMP1’ via chaperone ‘PfHsp70-x’”. In: ().
- [90] A G Maier et al. “Exported proteins required for virulence and rigidity of Plasmodium falciparum-infected human erythrocytes”. In: *Cell* 134.1 (2008), pp. 48–61.
- [91] Michael O Daniyan et al. “The Malarial Exported PFA0660w Is an Hsp40 Co-Chaperone of PfHsp70-x.” In: *PloS one* 11.2 (2016), e0148517.
- [92] Amita Dubey et al. “Heat shock proteins: a therapeutic target worth to consider.” In: *Veterinary world* 8.1 (2015), pp. 46–51.

- [93] Udai Banerji et al. "Phase I Pharmacokinetic and Pharmacodynamic Study of 17-Allylamino, 17-Demethoxygeldanamycin in Patients With Advanced Malignancies". In: *Journal of Clinical Oncology* 23.18 (2005), pp. 4152–4161.
- [94] Emmanuelle Tavernier et al. "HSP90 inhibition results in apoptosis of Philadelphia acute lymphoblastic leukaemia cells: an attractive prospect of new targeted agents". In: *Journal of Cancer Research and Clinical Oncology* 138.10 (2012), pp. 1753–1758.
- [95] Alessio Giubellino et al. "Targeting heat shock protein 90 for the treatment of malignant pheochromocytoma." In: *PloS one* 8.2 (2013). Ed. by Vladislav V. Glinskii, e56083.
- [96] Giulio Tosti et al. "Heat-shock proteins-based immunotherapy for advanced melanoma in the era of target therapies and immunomodulating agents." In: *Expert opinion on biological therapy* 14.7 (2014), pp. 955–67.
- [97] Qianli Meng, Bingbing X Li, and Xiangshu Xiao. "Toward Developing Chemical Modulators of Hsp60 as Potential Therapeutics." In: *Frontiers in molecular biosciences* 5 (2018), p. 35.
- [98] E-R Pesce et al. "Malaria heat shock proteins: drug targets that chaperone other drug targets." In: *Infectious disorders drug targets* 10.3 (2010), pp. 147–57.
- [99] Rajinder Kumar, Alla Musiyenko, and Sailen Barik. "The heat shock protein 90 of Plasmodium falciparum and antimalarial activity of its inhibitor, geldanamycin". In: *Malaria Journal* 2.1 (2003), p. 30.
- [100] Kacper B Rogala et al. "The Caenorhabditis elegans protein SAS-5 forms large oligomeric assemblies critical for centriole formation". In: *eLife* 4 (2015), e07410.
- [101] Raheem Ullah et al. "Activity of the Human Rhinovirus 3C Protease Studied in Various Buffers, Additives and Detergents Solutions for Recombinant Protein Production". In: *PLOS ONE* 11.4 (2016). Ed. by Mark J van Raaij, e0153436.
- [102] Elisabeth Gasteiger et al. *Protein Identification and Analysis Tools on the ExPaSy Server; (In) The Proteomics Protocols Handbook*. Ed. by John M Walker. Humana Press, 2005, pp. 571–607.
- [103] Frank Delaglio et al. "NMRPipe: A multidimensional spectral processing system based on UNIX pipes". In: *Journal of Biomolecular NMR* 6.3 (1995), pp. 277–293.
- [104] D Goddard, T; Kneller. *SPARKY 3*. Univeristy of Salifornia, San Francisco.
- [105] John R Taylor. *An introduction to error analysis*. 2nd. University Science Books, 1997.
- [106] Stephan Grzesiek and Ad Bax. "An efficient experiment for sequential backbone assignment of medium-sized isotopically enriched proteins". In: *Journal of Magnetic Resonance (1969)* 99.1 (1992), pp. 201–207.
- [107] Stephan Grzesiek and Ad Bax. "Correlating backbone amide and side chain resonances in larger proteins by multiple relayed triple resonance NMR". In: *Journal of the American Chemical Society* 114.16 (1992), pp. 6291–6293.
- [108] Aldino Viegas et al. "Saturation-Transfer Difference (STD) NMR: A Simple and Fast Method for Ligand Screening and Characterization of Protein Binding". In: *J. Chem. Educ* 88 (2011), pp. 990–994.

- [109] M R Webb. “A continuous spectrophotometric assay for inorganic phosphate and for measuring phosphate release kinetics in biological systems.” In: *Proceedings of the National Academy of Sciences of the United States of America* 89.11 (1992), pp. 4884–7.
- [110] Graeme Winter, Carina M C Lobley, and Stephen M Prince. “Decision making in xia2.” In: *Acta crystallographica. Section D, Biological crystallography* 69.Pt 7 (2013), pp. 1260–73.
- [111] Airlie J. McCoy et al. “Phaser crystallographic software”. In: *Journal of Applied Crystallography* 40.4 (2007), pp. 658–674.
- [112] Jonathan Pettinger et al. “An Irreversible Inhibitor of HSP72 that Unexpectedly Targets Lysine-56”. In: *Angewandte Chemie International Edition* 56.13 (2017), pp. 3536–3540.
- [113] P. Emsley et al. “Features and development of Coot”. In: *Acta Crystallographica Section D: Biological Crystallography* 66.4 (2010), pp. 486–501.
- [114] Thomas C. Terwilliger et al. “Iterative model building, structure refinement and density modification with the PHENIX AutoBuild wizard”. In: *Acta Crystallographica Section D Biological Crystallography* 64.1 (2008), pp. 61–69.
- [115] Vincent B. Chen et al. “MolProbity : all-atom structure validation for macromolecular crystallography”. In: *Acta Crystallographica Section D Biological Crystallography* 66.1 (2010), pp. 12–21.
- [116] Wolfgang Kabsch. “Integration, scaling, space-group assignment and post-refinement”. In: *Acta Crystallographica Section D Biological Crystallography* 66.2 (2010), pp. 133–144.
- [117] Clemens Vonnrhein et al. “Data processing and analysis with the autoPROC toolbox”. In: *Acta Crystallographica Section D Biological Crystallography* 67.4 (2011), pp. 293–302.
- [118] Marie-Françoise Incardona et al. “EDNA : a framework for plugin-based applications applied to X-ray experiment online data analysis”. In: *Journal of Synchrotron Radiation* 16.6 (2009), pp. 872–879.
- [119] Hyun Young Yu et al. “Roles of Intramolecular and Intermolecular Interactions in Functional Regulation of the Hsp70 J-protein Co-Chaperone Sis1”. In: *Journal of Molecular Biology* 427.7 (2015), pp. 1632–1643.
- [120] Andrew Waterhouse et al. “SWISS-MODEL: homology modelling of protein structures and complexes”. In: *Nucleic Acids Research* 46.W1 (2018), W296–W303.
- [121] Hamid R. Eghbalnia et al. “Protein energetic conformational analysis from NMR chemical shifts (PECAN) and its use in determining secondary structural elements”. In: *Journal of Biomolecular NMR* 32.1 (2005), pp. 71–81.
- [122] Kirsten Moll et al. “METHODS IN MALARIA RESEARCH”. In: ().
- [123] Parichat Prommana et al. “Inducible Knockdown of Plasmodium Gene Expression Using the glmS Ribozyme”. In: *PLoS ONE* 8.8 (2013). Ed. by Alister G. Craig, e73783.
- [124] W L DeLano. *The PyMOL Molecular Graphics System (2002) DeLano Scientific, Palo Alto, CA, USA. <http://www.pymol.org>. 2002.*

- [125] Manish Grover et al. "Identification of an exported heat shock protein 70 in *Plasmodium falciparum*". In: *Parasite* 20 (2013), p. 2.
- [126] V Arndt, C Rogon, and J Höhfeld. "To be, or not to be—molecular chaperones in protein degradation." In: *Cellular and molecular life sciences : CMLS* 64.19-20 (2007), pp. 2525–41.
- [127] L B Barradell and A Fitton. "Artesunate. A review of its pharmacology and therapeutic efficacy in the treatment of malaria." In: *Drugs* 50.4 (1995), pp. 714–41.
- [128] Rodolfo Thomé et al. "Chloroquine: Modes of action of an undervalued drug". In: *Immunology Letters* 153.1-2 (2013), pp. 50–57.
- [129] Abdoulaye Djimdé and Gilbert Lefèvre. "Understanding the pharmacokinetics of Coartem®". In: *Malaria Journal* 8.Suppl 1 (2009), S4.
- [130] Nuno Vale, Rui Moreira, and Paula Gomes. "Primaquine revisited six decades after its discovery". In: *European Journal of Medicinal Chemistry* 44.3 (2009), pp. 937–953.
- [131] Ingrid L Cockburn et al. "Screening for small molecule modulators of Hsp70 chaperone activity using protein aggregation suppression assays: inhibition of the plasmodial chaperone PfHsp70-1." In: *Biological chemistry* 392.5 (2011), pp. 431–8.
- [132] L Cockburn Ingrid et al. *Selective modulation of plasmodial Hsp70s by small molecules with antimalarial activity*. 2014.
- [133] Maria Rebelo et al. "A Novel Flow Cytometric Hemozoin Detection Assay for Real-Time Sensitivity Testing of *Plasmodium falciparum*". In: *PLoS ONE* 8.4 (2013). Ed. by Steffen Borrmann, e61606.
- [134] J. M. (J. Murray) Longmore. *Oxford handbook of clinical medicine, 8th ed. ; Oxford assess and progress clinical specialties*. Oxford University Press, 2012.
- [135] Srikanth Patury, Yoshinari Miyata, and Jason E Gestwicki. "Pharmacological targeting of the Hsp70 chaperone." In: *Current topics in medicinal chemistry* 9.15 (2009), pp. 1337–51.
- [136] Christopher G. Evans, Lyra Chang, and Jason E. Gestwicki. "Heat Shock Protein 70 (Hsp70) as an Emerging Drug Target". In: *Journal of Medicinal Chemistry* 53.12 (2010), pp. 4585–4602.
- [137] R Schlecht et al. "Functional analysis of Hsp70 inhibitors". eng. In: *PLoS One* 8.11 (2013), e78443.
- [138] Dick D Mosser and Richard I Morimoto. "Molecular chaperones and the stress of oncogenesis". In: *Oncogene* 23.16 (2004), pp. 2907–2918.
- [139] Sarah A. Broadley and F. Ulrich Hartl. "The role of molecular chaperones in human misfolding diseases". In: *FEBS Letters* 583.16 (2009), pp. 2647–2653.
- [140] Louis H. Miller et al. "The pathogenic basis of malaria". In: *Nature* 415.6872 (2002), pp. 673–679.
- [141] D Kwiatkowski. "Febrile temperatures can synchronize the growth of *Plasmodium falciparum* in vitro." In: *The Journal of experimental medicine* 169.1 (1989), pp. 357–61.

- [142] Kovit Pattanapanyasat et al. “Febrile temperature but not proinflammatory cytokines promotes phosphatidylserine expression on Plasmodium falciparum malaria-infected red blood cells during parasite maturation”. In: *Cytometry Part A* 77A.6 (2010), pp. 515–523.
- [143] Marina Marinkovic et al. “Febrile temperature leads to significant stiffening of Plasmodium falciparum parasitized erythrocytes”. In: *American Journal of Physiology-Cell Physiology* 296.1 (2009), pp. C59–C64.
- [144] Thomas D. Otto et al. “New insights into the blood-stage transcriptome of Plasmodium falciparum using RNA-Seq”. In: *Molecular Microbiology* 76.1 (2010), pp. 12–24.
- [145] Evgeny Krissinel and Kim Henrick. “Inference of Macromolecular Assemblies from Crystalline State”. In: *Journal of Molecular Biology* 372.3 (2007), pp. 774–797.
- [146] Vincent B. Chen et al. “MolProbity : all-atom structure validation for macromolecular crystallography”. In: *Acta Crystallographica Section D Biological Crystallography* 66.1 (2010), pp. 12–21.
- [147] K. Stierand, P. C. Maass, and M. Rarey. “Molecular complexes at a glance: automated generation of two-dimensional complex diagrams”. In: *Bioinformatics* 22.14 (2006), pp. 1710–1716.
- [148] M Shida et al. “Direct inter-subdomain interactions switch between the closed and open forms of the Hsp70 nucleotide-binding domain in the nucleotide-free state”. eng. In: *Acta Crystallogr D Biol Crystallogr* 66.Pt 3 (2010), pp. 223–232.
- [149] Maria Adell et al. “The nucleotide-bound/substrate-bound conformation of the Mycoplasma genitalium DnaK chaperone”. In: *Protein Science* 27.5 (2018), pp. 1000–1007.
- [150] Ming Yan, Jingzhi Li, and Bingdong Sha. “Structural analysis of the Sil1-Bip complex reveals the mechanism for Sil1 to function as a nucleotide-exchange factor.” In: *The Biochemical journal* 438.3 (2011), pp. 447–55.
- [151] Roman Kityk et al. “Structure and Dynamics of the ATP-Bound Open Conformation of Hsp70 Chaperones”. In: *Molecular Cell* 48.6 (2012), pp. 863–874.
- [152] Dirk Brehmer et al. “Tuning of chaperone activity of Hsp70 proteins by modulation of nucleotide exchange.” In: *Nature Structural Biology* 8.5 (2001), pp. 427–432.
- [153] S M Wilbanks, C DeLuca-Flaherty, and D B McKay. “Structural basis of the 70-kilodalton heat shock cognate protein ATP hydrolytic activity. I. Kinetic analyses of active site mutants.” In: *The Journal of biological chemistry* 269.17 (1994), pp. 12893–8.
- [154] K M Flaherty et al. “Structural basis of the 70-kilodalton heat shock cognate protein ATP hydrolytic activity. II. Structure of the active site with ADP or ATP bound to wild type and mutant ATPase fragment.” In: *The Journal of biological chemistry* 269.17 (1994), pp. 12899–907.
- [155] H Sonderrmann et al. “Structure of a Bag/Hsc70 complex: convergent functional evolution of Hsp70 nucleotide exchange factors.” In: *Science (New York, N.Y.)* 291.5508 (2001), pp. 1553–7.

- [156] C J Harrison et al. "Crystal structure of the nucleotide exchange factor GrpE bound to the ATPase domain of the molecular chaperone DnaK." In: *Science (New York, N.Y.)* 276.5311 (1997), pp. 431–5.
- [157] S Polier et al. "Structural basis for the cooperation of Hsp70 and Hsp110 chaperones in protein folding". eng. In: *Cell* 133.6 (2008), pp. 1068–1079.
- [158] Akash Bhattacharya et al. "Allostery in Hsp70 Chaperones Is Transduced by Subdomain Rotations". In: *Journal of Molecular Biology* 388.3 (2009), pp. 475–490.
- [159] Matthew Revington, Tina M Holder, and Erik R P Zuiderweg. "NMR study of nucleotide-induced changes in the nucleotide binding domain of Thermus Thermophilus Hsp70 chaperone DnaK: implications for the allosteric mechanism Running Title:Nucleotide-induced Structural Changes in DnaK". In: *JBC Papers in Press. Published on June Oxford Univ on July 2*.12 (2004).
- [160] Matthew Revington et al. "NMR Investigations of Allosteric Processes in a Two-domain Thermus thermophilus Hsp70 Molecular Chaperone". In: *Journal of Molecular Biology* 349.1 (2005), pp. 163–183.
- [161] Jeung-Hoi Ha and David B. McKay. "Kinetics of Nucleotide-Induced Changes in the Tryptophan Fluorescence of the Molecular Chaperone Hsc70 and Its Subfragments Suggest the ATP-Induced Conformational Change Follows Initial ATP Binding". In: *Biochemistry* 34.36 (1995), pp. 11635–11644.
- [162] Hyung-June Woo et al. "ATP-Induced Conformational Changes in Hsp70: Molecular Dynamics and Experimental Validation of an in Silico Predicted Conformation". In: *Biochemistry* 48.48 (2009), pp. 11470–11477.
- [163] J Yang et al. "Close and Allosteric Opening of the Polypeptide-Binding Site in a Human Hsp70 Chaperone BiP." In: *Structure* 23 (2015), pp. 2191–2203.
- [164] Radhey S. Gupta and Bhag Singh. "Phylogenetic analysis of 70 kD heat shock protein sequences suggests a chimeric origin for the eukaryotic cell nucleus". In: *Current Biology* 4.12 (1994), pp. 1104–1114.
- [165] S Lindquist and E A Craig. "The Heat-Shock Proteins". In: *Annual Review of Genetics* 22.1 (1988), pp. 631–677.
- [166] C Hunt and R I Morimoto. "Conserved features of eukaryotic hsp70 genes revealed by comparison with the nucleotide sequence of human hsp70." In: *Proceedings of the National Academy of Sciences of the United States of America* 82.19 (1985), pp. 6455–9.
- [167] P Lopez-Buesa, C Pfund, and E A Craig. "The biochemical properties of the ATPase activity of a 70-kDa heat shock protein (Hsp70) are governed by the C-terminal domains." In: *Proceedings of the National Academy of Sciences of the United States of America* 95.26 (1998), pp. 15253–8.
- [168] K Liberek et al. "Escherichia coli DnaJ and GrpE heat shock proteins jointly stimulate ATPase activity of DnaK." In: *Proceedings of the National Academy of Sciences of the United States of America* 88.7 (1991), pp. 2874–8.
- [169] D M Cyr, X Lu, and M G Douglas. "Regulation of Hsp70 function by a eukaryotic DnaJ homolog." In: *The Journal of biological chemistry* 267.29 (1992), pp. 20927–31.

- [170] L Otvos et al. "Interaction between heat shock proteins and antimicrobial peptides." In: *Biochemistry* 39.46 (2000), pp. 14150–9.
- [171] Jeffrey L Brodsky and Gabriela Chiosis. "Hsp70 molecular chaperones: emerging roles in human disease and identification of small molecule modulators." In: *Current topics in medicinal chemistry* 6.11 (2006), pp. 1215–25.
- [172] Yoshinari Miyata et al. "Cysteine Reactivity Distinguishes Redox Sensing by the Heat-Inducible and Constitutive Forms of Heat Shock Protein 70". In: *Chemistry & Biology* 19.11 (2012), pp. 1391–1399.
- [173] Alassane Dicko et al. "Efficacy and safety of primaquine and methylene blue for prevention of *Plasmodium falciparum* transmission in Mali: a phase 2, single-blind, randomised controlled trial". In: *The Lancet Infectious Diseases* 18.6 (2018), pp. 627–639.
- [174] G. Lu et al. "Efficacy and safety of methylene blue in the treatment of malaria: a systematic review". In: *BMC Medicine* 16.1 (2018), p. 59.
- [175] Augustin Zoungrana et al. "Safety and Efficacy of Methylene Blue Combined with Artesunate or Amodiaquine for Uncomplicated *Falciparum* Malaria: A Randomized Controlled Trial from Burkina Faso". In: *PLoS ONE* 3.2 (2008). Ed. by Aric Gregson, e1630.
- [176] S Wisen et al. "Binding of a small molecule at a protein-protein interface regulates the chaperone activity of hsp70-hsp40". In: *ACS Chem Biol* 5.6 (2010), pp. 611–622.
- [177] J Amick et al. "Crystal structure of the nucleotide-binding domain of mortalin, the mitochondrial Hsp70 chaperone". eng. In: *Protein Sci* 23.6 (2014), pp. 833–842.
- [178] Xiaokai Li et al. "Analogues of the Allosteric Heat Shock Protein 70 (Hsp70) Inhibitor, MKT-077, As Anti-Cancer Agents". In: (2013).
- [179] Aikaterini Rousaki et al. "Allosteric Drugs: The Interaction of Antitumor Compound MKT-077 with Human Hsp70 Chaperones". In: *Journal of Molecular Biology* 411.3 (2011), pp. 614–632.
- [180] Weizhong Li et al. "The EMBL-EBI bioinformatics web and programmatic tools framework." In: *Nucleic acids research* 43.W1 (2015), W580–4.
- [181] Kazutaka Katoh, George Asimenos, and Hiroyuki Toh. "Multiple Alignment of DNA Sequences with MAFFT". In: Humana Press, 2009, pp. 39–64.
- [182] Judith H. Prieto. "The Binding of Methylene Blue to *Plasmodium Falciparum* Glutathione Reductase". In: *Biophysical Journal* 112.3 (2017), 351a.
- [183] Katharina Ehrhardt et al. "The antimalarial activities of methylene blue and the 1,4-naphthoquinone 3-[4-(trifluoromethyl)benzyl]-menadione are not due to inhibition of the mitochondrial electron transport chain." In: *Antimicrobial agents and chemotherapy* 57.5 (2013), pp. 2114–20.
- [184] X. B. Qiu et al. *The diversity of the DnaJ/Hsp40 family, the crucial partners for Hsp70 chaperones*. 2006.
- [185] O Deloche, W L Kelley, and C Georgopoulos. "Structure-function analyses of the Ssc1p, Mdj1p, and Mge1p *Saccharomyces cerevisiae* mitochondrial proteins in *Escherichia coli*." In: *Journal of bacteriology* 179.19 (1997), pp. 6066–75.

- [186] Pierre Genevaux et al. “DjlA Is a Third DnaK Co-chaperone of Escherichia coli , and DjlA-mediated Induction of Colanic Acid Capsule Requires DjlA-DnaK Interaction”. In: *Journal of Biological Chemistry* 276.11 (2001), pp. 7906–7912.
- [187] Christoph J. Kluck et al. “Structure-Function Analysis of HscC, the Escherichia coli Member of a Novel Subfamily of Specialized Hsp70 Chaperones”. In: *Journal of Biological Chemistry* 277.43 (2002), pp. 41060–41069.
- [188] Jingzhi Li, Xinguo Qian, and Bingdong Sha. “Heat shock protein 40: structural studies and their functional implications.” In: *Protein and peptide letters* 16.6 (2009), pp. 606–12.
- [189] Collaborative Computational Project, Number 4. “The CCP4 suite: programs for protein crystallography”. In: *Acta Crystallographica Section D Biological Crystallography* 50.5 (1994), pp. 760–763.
- [190] Paul D. Adams et al. “PHENIX : a comprehensive Python-based system for macromolecular structure solution”. In: *Acta Crystallographica Section D Biological Crystallography* 66.2 (2010), pp. 213–221.
- [191] Joyce Tsai and Michael G. Douglas. “A Conserved HPD Sequence of the J-domain Is Necessary for YDJ1 Stimulation of Hsp70 ATPase Activity at a Site Distinct from Substrate Binding”. In: *Journal of Biological Chemistry* 271.16 (1996), pp. 9347–9354.
- [192] M. Pellicchia et al. “NMR structure of the J-domain and the Gly/Phe-rich region of the Escherichia coli DnaJ chaperone.” In: *J.Mol.Biol.* 260 (1996), pp. 236–250.
- [193] J. Osipiuk et al. “X-ray crystal structure of J-domain of dnj-12 from Caenorhabditis elegans.” In: *TO BE PUBLISHED* ().
- [194] Y.Q. Qian et al. “Nuclear magnetic resonance solution structure of the human Hsp40 (HDJ-1) J-domain.” In: *J.Mol.Biol.* 260 (1996), pp. 224–235.
- [195] J. Jiang et al. “Structure-function analysis of the auxilin J-domain reveals an extended Hsc70 interaction interface.” In: *Biochemistry* 42 (2003), pp. 5748–5753.
- [196] Pierre Genevaux et al. “Scanning Mutagenesis Identifies Amino Acid Residues Essential for the in Vivo Activity of the Escherichia coli DnaJ (Hsp40) J-Domain”. In: *Genetics* 162.3 (2002).
- [197] Fritha Hennessy, Aileen Boshoff, and Gregory L. Blatch. “Rational mutagenesis of a 40 kDa heat shock protein from Agrobacterium tumefaciens identifies amino acid residues critical to its in vivo function”. In: *The International Journal of Biochemistry & Cell Biology* 37.1 (2005), pp. 177–191.
- [198] H.Y. Yu et al. “Roles of intramolecular and intermolecular interactions in functional regulation of the Hsp70 J-protein co-chaperone Sis1.” In: *J.Mol.Biol.* 427 (2015), pp. 1632–1643.
- [199] Andrew Waterhouse et al. “SWISS-MODEL: homology modelling of protein structures and complexes”. In: *Nucleic Acids Research* (2018).
- [200] Roman Kityk, Jürgen Kopp, and Matthias P. Mayer. “Molecular Mechanism of J-Domain-Triggered ATP Hydrolysis by Hsp70 Chaperones”. In: *Molecular Cell* (2017).

- [201] Samuel J. Landry. “Structure and Energetics of an Allele-Specific Genetic Interaction between dnaJ and dnaK : Correlation of Nuclear Magnetic Resonance Chemical Shift Perturbations in the J-Domain of Hsp40/DnaJ with Binding Affinity for the ATPase Domain of Hsp70/DnaK”. In: *Biochemistry* 42.17 (2003), pp. 4926–4936.
- [202] W H Wernsdorfer. “The development and spread of drug-resistant malaria.” In: *Parasitology today (Personal ed.)* 7.11 (1991), pp. 297–303.
- [203] Shweta Sinha, Bikash Medhi, and Rakesh Sehgal. “Challenges of drug-resistant malaria”. In: *Parasite* 21 (2014), p. 61.
- [204] Arjen M. Dondorp et al. “The Threat of Artemisinin-Resistant Malaria”. In: *New England Journal of Medicine* 365.12 (2011), pp. 1073–1075.
- [205] M. Enserink. “Malaria’s Drug Miracle in Danger”. In: *Science* 328.5980 (2010), pp. 844–846.
- [206] World Health Organization. *Artemisinin resistance and artemisinin-based combination therapy efficacy (Status report – August 2018)*. Tech. rep.
- [207] Henri Vial et al. “CRIMALDDI: platform technologies and novel anti-malarial drug targets”. In: *Malaria Journal* 12.1 (2013), p. 396.
- [208] Yun Chen et al. “Repurposing drugs to target the malaria parasite unfolding protein response”. In: *Scientific Reports* 8.1 (2018), p. 10333.
- [209] Hiba Abi Hussein et al. “PockDrug-Server: a new web server for predicting pocket druggability on holo and apo proteins”. In: *Nucleic Acids Research* 43.W1 (2015), W436–W442.
- [210] Liisa Holm and Laura M Laakso. “Dali server update.” In: *Nucleic acids research* 44.W1 (2016), W351–5.
- [211] A Arakawa et al. “Biochemical and structural studies on the high affinity of Hsp70 for ADP”. eng. In: *Protein Sci* 20.8 (2011), pp. 1367–1379.
- [212] Ziming Zhang et al. “New crystal structures of HSC-70 ATP binding domain confirm the role of individual binding pockets and suggest a new method of inhibition”. In: *Biochimie* 108 (2015), pp. 186–192.
- [213] Elizabeth A. Craig and Jaroslaw Marszalek. “How Do J-Proteins Get Hsp70 to Do So Many Different Things?” In: *Trends in Biochemical Sciences* 42.5 (2017), pp. 355–368.
- [214] Fritha Hennessy et al. “Not all J domains are created equal: implications for the specificity of Hsp40-Hsp70 interactions.” In: *Protein science : a publication of the Protein Society* 14.7 (2005), pp. 1697–709.
- [215] Harm H. Kampinga and Elizabeth A. Craig. “The HSP70 chaperone machinery: J proteins as drivers of functional specificity”. In: *Nature Reviews Molecular Cell Biology* 11.8 (2010), pp. 579–592.
- [216] May Bakail and Francoise Ochsenbein. “Targeting protein–protein interactions, a wide open field for drug design”. In: *Comptes Rendus Chimie* 19.1-2 (2016), pp. 19–27.

- [217] M Botha, E R Pesce, and G L Blatch. “The Hsp40 proteins of *Plasmodium falciparum* and other apicomplexa: regulating chaperone power in the parasite and the host”. In: *Int J Biochem Cell Biol* 39.10 (2007), pp. 1781–1803.
- [218] Pragyan Acharya et al. “An Exported Heat Shock Protein 40 Associates with Pathogenesis-Related Knobs in *Plasmodium falciparum* Infected Erythrocytes”. In: *PLoS ONE* 7.9 (2012). Ed. by Gordon Langsley, e44605.
- [219] Jan D. Warncke, Ioannis Vakonakis, and Hans-Peter Beck. “*Plasmodium* Helical Interspersed Subtelomeric (PHIST) Proteins, at the Center of Host Cell Remodeling”. In: *Microbiology and Molecular Biology Reviews* 80.4 (2016), pp. 905–927.
- [220] Monica Diez Silva et al. “A role for the *Plasmodium falciparum* RESA protein in resistance against heat shock demonstrated using gene disruption”. In: *Molecular Microbiology* 56.4 (2005), pp. 990–1003.
- [221] X. Pei et al. “The ring-infected erythrocyte surface antigen (RESA) of *Plasmodium falciparum* stabilizes spectrin tetramers and suppresses further invasion”. In: *Blood* 110.3 (2007), pp. 1036–1042.
- [222] Casilda G. Black et al. “In vivo studies support the role of trafficking and cytoskeletal-binding motifs in the interaction of MESA with the membrane skeleton of *Plasmodium falciparum*-infected red blood cells”. In: *Molecular and Biochemical Parasitology* 160.2 (2008), pp. 143–147.
- [223] Gowrishankar Banumathy, Varsha Singh, and Utpal Tatu. “Host Chaperones Are Recruited in Membrane-bound Complexes by *Plasmodium falciparum*”. In: *Journal of Biological Chemistry* 277.6 (2002), pp. 3902–3912.
- [224] Angelo D ’ Alessandro, Pier Giorgio Righetti, and Lello Zolla. “The Red Blood Cell Proteome and Interactome: An Update”. In: ().