Structural-functional diversity of malaria parasite’s PfHSP70-1 and PfHSP40 chaperone pair gives an edge over human orthologs in chaperone-assisted protein folding.

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Abstract

*Plasmodium falciparum*, the human malaria parasite harbors a metastable proteome which is vulnerable to proteotoxic stress conditions encountered during its lifecycle. How parasite’s chaperone machinery is able to maintain its aggregation-prone proteome in functional state, is poorly understood. As HSP70-40 system forms the central hub in cellular proteostasis, we investigated the protein folding capacity of PfHSP70-1 and PfHSP40 chaperone pair and compared it with human orthologs (HSPA1A and DNAJA1). Despite structural similarity, we observed that parasite chaperones and their human orthologs exhibit striking differences in conformational dynamics. Comprehensive biochemical investigations revealed that PfHSP70-1 and PfHSP40 chaperone pair has better protein folding, aggregation inhibition and oligomer remodeling and disaggregate activities than their human orthologs. Chaperone-swapping experiments suggest that PfHSP40 can also efficiently cooperate with human HSP70 to facilitate folding of client-substrate. SPR-derived kinetic parameters reveal that PfHSP40 has higher binding affinity towards unfolded substrate than DNAJA1. Interestingly, the observed slow dissociation rate of PfHSP40-substrate interaction allows PfHSP40 to maintain substrate in folding-competent state to minimize its misfolding. Structural investigation through SAXS gave insights into the conformational architecture of PfHSP70-1 (monomer), PfHSP40 (dimer) and their complex. Overall, our data suggests that parasite has evolved functionally diverged and efficient chaperone machinery which allows human malaria parasite to survive in hostile conditions. The distinct allosteric landscapes and interaction kinetics of plasmodial chaperones open avenues for exploration of small-molecule based antimalarial interventions.
Introduction

Human malaria parasite encounters frequent environmental fluctuations (pH, nutrients, temperature, oxygen) and immune threats as it homes different tissues; and transits through warm-blooded host (human for asexual cycle) and cold blooded vector (mosquito for sexual cycle) [1]. Amongst the malaria causing Plasmodium spp., P. falciparum has acquired striking proteome features that are distinct from other species. Its proteome harbors low complexity regions which have high frequency of asparagine and glutamines homorepeats [2-4]. Homorepeats impart prion-like features or disorder which allows proteins to undergo dynamic conformational transitions for protein-protein interactions [5, 6]. However, this structural-functional advantage of a proteome enriched with disordered domains has a trade-off associated with thermodynamic and kinetic constrains in protein folding [7]. Hence, acquisition of homorepeats in a proteome is inter-linked with co-acquisition of a robust proteostasis machinery [8, 9]. This machinery involves close cooperation between protein folding and degradation processes to limit cellular protein misfolding and/or coordinate efficient disaggregation or degradation of misfolded proteins.

Phylogenetic analysis of P. falciparum has revealed that its proteostasis machinery has significantly diverged from its human orthologs, and few components even exhibit species-specific sequence variations which may promote auxiliary interactions to give survival advantage to the parasite during hostile microenvironments [10, 11]. During protein synthesis in Plasmodium, frequent environmental perturbations may interfere with correct folding of nascent polypeptide, its targeting to subcellular compartments and export of parasite’s cargo. The cellular chaperones form the first-line of defense by interacting with nascent polypeptide to ensure that it has attained its functional state and is properly targeted to the destined cellular organelle. The chaperone network primarily includes ribosome-bound chaperones, sHSPs, HSP40, HSP70, HSP60, HSP90 and HSP110 [12-17]. In the cellular protein folding cascade, HSP70-HSP40 forms the central hub which facilitates co- and post-translational folding of nascent polypeptides [12, 13]. It also interacts with the disaggregate and degradation machinery to coordinate refolding of misfolded polypeptides or efficient removal of terminally aggregated species [18-21]. HSP70 family is ubiquitous and highly conserved in different organisms, while its co-chaperone HSP40 family comprise of structurally and functionally diverse proteins which drive substrate specificity to HSP70-mediated protein folding and disaggregation [22, 23]. Plasmodial HSP70s (4 isoforms) and HSP40s (45 isoforms) are localized in distinct sub-cellular compartments (cytosol, nucleus, apicoplast, mitochondrion, ER) [24-28]. They are even exported into human RBC to facilitate trafficking of parasite cargo proteins to sequester nutrients from host, export of virulence factors (PfEMP1) via transient cellular organelles onto RBC surface and modulate the repertoire of client proteins for immune evasion [29-32].
Cooperation of HSP70-HSP40 has been extensively investigated for bacterial, yeast and human orthologs [33-37]. Studies have revealed key variations in the interaction interfaces between HSP70-HSP40 and client proteins; and this fine tunes HSP70-HSP40 activity in different organisms [20, 37-39]. Such biochemical and structural investigations for *P. falciparum* chaperones are lacking due to challenges associated with poor protein expression related to codon bias, solubility, concentration-dependent oligomer formation and proteolysis issues. Till date, biochemical studies for parasite chaperones have been limited to ATPase activity and suppression of aggregation [40-44]. Plasmodial HSP70-HSP40 have also been suggested to be potential drug targets [45, 46]. However, this exploration requires thorough structural-functional characterization and comparison with human orthologs to minimize off-target effects in antimalarial interventions. In this study, we have performed comprehensive biochemical investigations (conformational dynamics, refolding, aggregation inhibition, oligomer remodeling, disaggregase activity and interaction kinetics) for plasmodial HSP70-40 (PfHSP70-1, type-I PfHSP40) machinery and compared it with their human orthologs. In addition, we provide the insights into the shape and conformation of PfHSP70-1, PfHSP40 and their complex. Our data suggests that parasite has evolved an efficient PfHSP70-1 and PfHSP40 system which supports parasite survival in human host. These findings open avenues for rational small-molecule based SAR for disruption of critical parasite chaperone interactions for antimalarial interventions.

**Materials and Methods**

*Preparation of constructs*

PfHSP70-1 (PF3D7_0818900), PfHSP40 (PF3D7_1437900), HSPA1A (P0DMV8) and DNAJA1 (P31689) were cloned in pET21d. PfHSP40 and PfHSP70-1 genes were amplified from PF3D7 cDNA and genomic DNA, respectively. DNAJA1 and HSPA1A were amplified from cDNA prepared from HEK293T. The detail of primers is provided in supporting information (Supplementary Table S1).

*Purification of Plasmodial and human chaperones*

PfHSP40 (PF3D7_1437900): pET21d-PfHSP40 and RIG plasmid (Baca and Hol, 2000, Int. J. Parasitol.) were co-transformed into BL21(DE3). The culture was grown in AIM media supplemented with ampicillin (25 µg/mL) and chloramphenicol (12 µg/mL) at 37 °C for 4 h. At OD600 of 0.8, the culture was shifted to 25 °C for 16 h. Cells were harvested by spinning at 5000 rpm for 10 mins at 4 °C. Pellet was resuspended in lysis buffer (50 mM Tris pH 8.0, 5 mM MgCl2, 500 mM KCl, 10% glycerol, 10 mM imidazole, 1 mM β-mercaptoethanol, 0.2% Triton X-100, 0.2% NP40 and 1 mM PMSF). Cells were sonicated and lysate was clarified by centrifugation at 9000 rpm for 25 mins at 4 °C. Supernatant was
loaded on pre-equilibrated Ni-NTA beads (Qiagen) and incubated for 2 h at 4 °C. The flow through was collected and beads were washed with 200 mL wash buffer (50 mM Tris pH 8.0, 5 mM MgCl₂, 500 mM KCl, 10 % glycerol, 40 mM imidazole, 1 mM β-mercaptoethanol). Protein was eluted with elution buffer (50 mM Tris pH 8.0, 5 mM MgCl₂, 500 mM KCl, 10% glycerol, 300 mM imidazole, 1 mM β-mercaptoethanol) and buffer exchange was performed with buffer B (50 mM Tris pH 8.0, 5 mM MgCl₂, 120 mM KCl, 5% glycerol) before loading on anion exchange column (HiTrap® Q Fast Flow GE Healthcare, 5mL). The protein was eluted using a linear gradient of KCl (120 mM to 1 M). The eluted protein was loaded on S200 increase 10/300 (GE healthcare), pre-equilibrated with buffer (50 mM Tris pH 8.0, 5 mM MgCl₂, 300 mM KCl, 2 % glycerol and 1 mM β-mercaptoethanol). Elution peak corresponding to dimer was collected and supplemented with 10% glycerol for storage in -80 °C.

PfHSP70-1 (PF3D7_0818900): pET21d-PfHSP70-1 was transformed into Rosetta™(DE3) and the culture was grown in TB media supplemented with ampicillin (25 µg/mL) and chloramphenicol (12 µg/mL) at 37 °C. At OD₆₀₀ of 0.6, culture was induced with 0.3 mM IPTG and was grown at 25 °C, 16 h. Culture was pelleted at 5000 rpm for 10 mins and then resuspended in lysis buffer (50 mM Tris pH 8.0, 5 mM MgCl₂, 500 mM KCl, 10 % glycerol, 10 mM imidazole, 5 mM β-mercaptoethanol, 0.25 % NP40 and 1 mM PMSF). The culture was sonicated and lysate was clarified at 9000 rpm for 25 mins at 4 °C. The supernatant was incubated with pre-equilibrated Ni-NTA beads (Qiagen) for 2 h at 4 °C. The flow through was collected and beads were washed with 200 mL wash buffer (50 mM Tris pH 8.0, 5 mM MgCl₂, 500 mM KCl, 10 % glycerol, 50 mM imidazole, 5 mM β-mercaptoethanol). Protein was eluted in elution buffer (50 mM Tris pH 8.0, 5 mM MgCl₂, 500 mM KCl, 10 % glycerol, 50 mM imidazole, 5 mM β-mercaptoethanol). Eluted protein was loaded on S200 increase 10/300 column (GE healthcare), pre-equilibrated with buffer (50 mM Tris pH 8.0, 5 mM MgCl₂, 500 mM KCl, 2 % glycerol, 5 mM β-mercaptoethanol). Elution peak corresponding to monomer was collected and supplemented with 10% glycerol for storage in -80 °C. We observe a degradation band in PfHSP70-1 protein which could not be removed through different purification steps.

DNAJA1 (P31689): pET21d-DNAJA1 was transformed into BL21(DE3). Culture was grown in TB media supplemented with ampicillin (25 µg/mL) and incubated at 37 °C. At OD₆₀₀ of 0.6, culture was induced with 0.5 mM IPTG and was grown at 25 °C for 16 h. Culture was harvested at 5000 rpm for 10 mins at 4 °C. Pellet was resuspended in lysis buffer (50 mM Tris pH 7.5, 5 mM MgCl₂, 500 mM KCl, 10 % glycerol, 10 mM imidazole, 1 mM β-mercaptoethanol, 0.25 % NP40 and 1 mM PMSF). Culture was sonicated and lysate was clarified by centrifugation at 9000 rpm for 25 mins at 4 °C. Supernatant was incubated with pre-equilibrated Ni-NTA beads (Qiagen) for 2 h at 4 °C. The flow through was collected and beads were washed with 200 mL wash buffer (50 mM Tris pH 7.5, 5 mM MgCl₂, 500 mM KCl, 10 %
glycerol, 30 mM imidazole, 1 mM β-mercaptoethanol). Protein was eluted in elution buffer (50 mM Tris pH 7.5, 5 mM MgCl₂, 500 mM KCl, 10 % glycerol, 400 mM imidazole, 1 mM β-mercaptoethanol). Purified protein was loaded on S200 increase 10/300 (GE Healthcare), pre-equilibrated with buffer (50 mM Tris pH 7.5, 5 mM MgCl₂, 500 mM KCl, 2 % glycerol, 1 mM β-mercaptoethanol). Elution peak corresponding to dimer was collected and supplemented with 10% glycerol for storage in -80 °C.

HSPA1A (P0DMV8): pET21-HSPA1A plasmid was transformed into BL21(DE3). Culture was grown in TB media containing ampicillin (25 µg/mL) at 37 °C. At OD₆₀₀ of 0.6, the culture was induced with 1 mM IPTG and incubated at 16 °C for 16 h. Cells were harvested at 5000 rpm and resuspended in lysis buffer (50 mM Tris pH 7.0, 5 mM MgCl₂, 500 mM KCl, 10 % glycerol, 10 mM imidazole, 5 mM β-mercaptoethanol, 0.25 % NP40 and 1 mM PMSF). Culture was sonicated and lysate was clarified by centrifugation at 9000 rpm for 25 mins at 4 °C. Supernatant was incubated with pre-equilibrated Ni-NTA beads (Qiagen) for 2 h at 4 °C. The flow through was collected and beads were washed with 200 mL wash buffer (50 mM Tris pH 7.0, 5 mM MgCl₂, 500 mM KCl, 10% glycerol, 40 mM imidazole, 5 mM β-mercaptoethanol). Protein was eluted in elution buffer (50 mM Tris pH 7.0, 5 mM MgCl₂, 500 mM KCl, 10% glycerol, 400 mM imidazole, 5 mM β-mercaptoethanol). The protein was further loaded on S200 increase 10/300 (GE healthcare), pre-equilibrated with buffer (50 mM Tris pH 7.0, 5 mM MgCl₂, 500 mM KCl, 2 % glycerol, 5 mM β-mercaptoethanol). Elution peak corresponding to monomer fraction was collected and supplemented with 10 % glycerol for storage at -80 °C.

**Purification of Luciferase and generation of polyclonal antibody**

pT7-luciferase (from Spirin’s lab, Svetlov et al, 2006, Protein Sci) was transformed in BL21(DE3) and culture was grown in TB media at 37 °C. At OD₆₀₀ of 0.6, culture was induced with 0.5 mM IPTG and incubated at 25 °C for 16 h. Cells were harvested and resuspended in lysis buffer (50 mM Tris, pH 7.5, 5 mM MgCl₂, 500 mM KCl, 10 % glycerol, 10 mM imidazole, 1 mM β-mercaptoethanol, 0.25 % NP40 and 1 mM PMSF). Culture was sonicated and lysate was clarified by centrifugation at 9000 rpm for 25 mins at 4 °C. Supernatant was incubated with pre-equilibrated Ni NTA beads (Qiagen) for 2 h at 4 °C. The flow through was collected and beads were washed with 200 mL wash buffer (50 mM Tris pH 7.5, 5 mM MgCl₂, 500 mM KCl, 10% glycerol, 40 mM imidazole, 1 mM β-mercaptoethanol). Protein was eluted in elution buffer (50 mM Tris pH 7.5, 5 mM MgCl₂, 500 mM KCl, 10% glycerol, 400 mM imidazole, 1 mM β-mercaptoethanol). Eluted protein was loaded on S200 increase 10/300 (GE healthcare), pre-equilibrated with buffer (50 mM Tris pH 7.5, 5 mM MgCl₂, 500 mM KCl, 2 % glycerol, 1 mM β-mercaptoethanol). Monomer (corresponding to 66 kDa) and oligomer (> 660 kDa) fractions were collected and were supplemented with 10 % glycerol for storage at -80 °C. The purified luciferase
monomer was used to raise polyclonal antibody. Purified luciferase protein (500 µg/mL) was mixed with Freund’s complete adjuvant (1:1) which was injected subcutaneously in to NZW female rabbit followed with booster 1 after 28 days of immunization. Booster 2 was given 14 days after the first booster dose. The booster dose is prepared with luciferase protein (500 µg/mL) mixed with Freund’s incomplete adjuvant. Blood was collected after ten days of second booster. Antisera was collected by processing the blood. Purified luciferase protein was run on SDS PAGE and transferred on to nitrocellulose membrane followed by blocking the membrane with 5% skimmed milk for 1 h at room temperature. Membrane was washed with 1× PBS and incubated with antisera (1:10) dilution 4 °C overnight. The blot was washed twice with 0.1 % PBST and into small pieces and put in a micro-centrifuge tube. Antibodies were eluted by addition of 500 µl glycine buffer (0.2 M Glycine-HCl pH 2.3, 500 mM NaCl); the eluate was immediately neutralized by the addition of 1 M Tris (pH 8.0) till the pH of the solution reached pH 7.4. This elution step was repeated three times. 10 % glycerol was added in eluted polyclonal antibody and stored at -80 °C. The specificity of luciferase antibody was compared with pre-immune sera against luciferase protein.

**Probing conformational dynamics in human and parasite chaperones**

Limited proteolysis experiments (using 2 µM trypsin, Sigma T6567) were performed with human (40 µM HSPA1A) and parasite chaperone (40 µM PfHSP70-1) in absence or presence of nucleotide (apo) and/or NR-peptide. Prior to trypsin digestion, parasite and human HSP70 was incubated with 5 mM ATP (Sigma A2383) or ADP (Sigma A2754) in absence or presence of 120 µM peptide (NR: NRLLLTG) in buffer (50 mM Tris pH 8.0, 5 mM MgCl2, 50 mM KCl, 5 mM β-mercaptoethanol) at 4 °C for 2 h. Subsequently, the sample was subjected to protease digestion at 25 °C and aliquots were taken at different time-points and immediately boiled in SDS loading dye. Trypsin digestion was also performed for human (40 µM DNAJA1) and parasite (40 µM PfHSP40) in absence and presence of 120 µM peptide (ter-70: GPTVEEVD). HSP40s were incubated with ter-70 peptide at 4 °C for 2 h followed by trypsin digestion at 25 °C. Aliquots were taken at different time-points and immediately boiled in SDS loading dye. The samples were run on SDS-PAGE and visualized through Commissie staining.

**Circular Dichroism measurements**

Circular Dichroism (CD) spectra were recorded for 10 µM plasmodial and human HSP70 and HSP40 orthologs. Samples were prepared in 50 mM HEPES pH 8.0, 200 mM NaF. Scans were recorded in the range of 200 nm-250 nm with increasing temperature (0.3 °C/min).
**ATPase Assay**

ATPase activity of parasite and human HSP70 was determined through Enzcheck phosphate assay kit (E6646). For a total reaction volume of 400 µl, parasite and human HSP70 (1 µM) and HSP40 (4 µM) were incubated in buffer X (50 mM Tris pH 7.5, 12 mM MgCl₂, 50 mM KCl, 2 mM β-mercaptoethanol) for 15 mins at 25 °C and 37 °C. This was followed by addition of 80 µl of 2-amino-6-mercapto-7-methylpurine riboside (MESG) substrate and 4 µl of purine nucleoside phosphorylase (PNP) (provided in the kit) with additional incubation for 15 mins at 25 °C and 37 °C. For control reaction, above mentioned procedure was followed with BSA (5 µM). Reactions were started by adding 2 mM ATP (Sigma A2383) and read-out for ATP hydrolysis was monitored at Abs360 nm using UV-vis spectrometer (Jasco V750) at 25 °C and 37 °C.

**Foldase, holdase and oligomer remodeling activities**

Foldase activity: Recombinant luciferase (2 mg/mL) was precipitated by adding five volumes acetone and centrifuge at 10000 rpm for 20 mins at 4 °C as described previously [47]. Pellet was resuspended in denaturation buffer (50 mM Tris pH 7.5, 50 mM KCl, 6 M Guanidine HCl, 2 mM β-mercaptoethanol) to prepare a stock of 30 µM. Denatured luciferase was diluted to a concentration of 0.3 µM into refolding buffer (50 mM Tris pH 7.5, 12 mM MgCl₂, 50 mM KCl, 2 mM β-mercaptoethanol) and supplemented with HSP40 (4 µM) and HSP70 (1 µM) and 3 mM ATP. Refolding was performed at 25 °C and 37 °C. Aliquots (30 µL) of refolding reactions were collected at different time-points to measure luminescence using 30 µL luciferase assay buffer (50 mM Tris pH 7.5, 15 mM MgCl₂, 2 mM ATP, 1 mM luciferin).

Holdase activity: Recombinant luciferase (0.6 µM) and HSP40 orthologs (8 µM) were incubated in refolding buffer (50 mM Tris pH 7.5, 12 mM MgCl₂, 50 mM KCl, 2 mM β-mercaptoethanol) at 42 °C for 10 mins. After incubation, this sample was diluted into equal volume of refolding buffer containing HSP70 (2 µM) and 3 mM ATP followed by incubation at 25 °C and 37 °C. Aliquots (30 µL) of sample were collected at different time-points to measure luminescence at 25 °C using 30 µL of luciferase assay buffer (50 mM Tris pH 7.5, 15 mM MgCl₂, 2 mM ATP, 1 mM luciferin). For control experiments for foldase and holdase activities, luminescence was monitored for chemically or thermally denatured luciferase in the absence of chaperones. In swapping experiments, chemically or thermally denatured luciferase was incubated with (a) 1 µM PfHSP70-1 and 4 µM DNAJA1 or (b) 1 µM HSPA1A and 4 µM PfHSP40. Luminescence was recorded in Flx800 from Biotek. Data is represented as % luciferase refolded obtained after normalization with luminescence from natively folded luciferase.
MDH assay: Malate dehydrogenase (MDH, Sigma M2634) was used as a substrate. MDH (0.6 µM) was denatured at 45 °C for 1 h in the presence and absence of 8 µM HSP40 (DNAJA1 or PfHSP40). After incubation, this sample was diluted into equal volume of refolding buffer (50 mM Tris pH 7.5, 12 mM MgCl₂, 50 mM KCl, 2 mM β-mercaptoethanol) containing 2 µM HSP70 (HSPA1A or PfHSP70-1), 3 mM phosphoenol pyruvate (Sigma P0564), 80 µM pyruvate kinase (Sigma P7768) and 5 mM ATP. Upon dilution into refolding buffer, the final concentrations were 0.3 µM MDH, 4 µM HSP40 and 1 µM HSP70. The activity of refolded MDH was assayed by adding substrate buffer (0.5 mM OAA and 0.25 mM NADH). OAA (O4126) and NADH (N8129) were procured from Sigma. NAD⁺ absorbance was taken at 340 nm using UV-vis spectrometer (Jasco V750).

Oligomer remodelling activity

Luciferase monomer (4 µg) and oligomer (4 µg) were incubated with BSA (5 µM) for 30 mins (25 °C), followed by trypsin (T6567, Sigma) digestion (2.5 µM) at 25 °C. In parallel, luciferase oligomer was incubated with parasite and human chaperone pair (1 µM HSP70, 4 µM HSP40) for 30 mins at 25 °C followed by trypsin digestion at 25 °C. All the experiments were performed in 50 mM Tris pH 7.5, 12 mM MgCl₂, 50 mM KCl, 2 mM β-mercaptoethanol. The samples were run on SDS-PAGE and visualized through western blotting with polyclonal luciferase antibody (1:1000 dilution) as primary antibody and anti-rabbit IgG HRP-conjugate antibody (Sigma A6154; 1:5000 dilution). Luciferase activity of oligomers was also monitored at different time-points upon incubation in absence and presence of chaperones (parasite and human HSP70-HSP40).

Disaggregase activity

Luciferase (30 µM) was incubated in denaturing buffer (50 mM Tris pH 7.5, 50 mM KCl, 8 M Urea, 2 mM β-mercaptoethanol) at 30 °C for 45 mins [48]. The aggregated luciferase was diluted 100 fold in refolding buffer (50 mM Tris pH 7.5, 12 mM MgCl₂, 50 mM KCl, 2 mM β-mercaptoethanol). Refolding buffer was supplemented with plasmodial and human HSP70 (1 µM), HSP40 (4 µM), 5mM PEP (Phosphoenol pyruvate), 10 U/mL pyruvate kinase and 5 mM ATP. Disaggregation of luciferase was performed at 25 °C and samples (30 µL) were collected at different time points. The disaggregation of luciferase was measured through luminescence using luciferase assay buffer (50 mM Tris pH 7.5, 15 mM MgCl₂, 2 mM ATP, 1 mM luciferin).
Surface Plasmon Resonance (SPR)

The SPR experiments were performed using BIAcore 3000 system (GE Healthcare) at 25 °C. The N-hydroxy succinimide (50 mM NHS) and N-ethyl-N-(diethylaminopropyl) carbodiimide (EDC, 0.2 M) was used to activate the CM5 sensor chip (BR-1000-12, GE healthcare). His-antibody (Santa Cruz AD1.1.10) was immobilized by amine coupling to the activated surface at concentration of 200 µg/mL in 10 mM sodium acetate buffer at pH 5.0 to a density of 8000 response units. After antibody immobilization, the surface was blocked using ethanolamine at pH 7.4 and 50 mM NaOH was used as regeneration solution. A second flow cell served as a reference cell to account for bulk-shift responses and minor, nonspecific interactions which was subtracted from the binding response. The PfHSP40 and DNAJA1 were diluted in 1x HBS-EP+ (BR-1006-69, GE healthcare; 0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005 % v/v Surfactant P20) and injected as a ligand (concentration of 250 nM) at a flow rate of 5 µl/min. Luciferase (SRE0045, Sigma) was used as analyte in the experiment. Luciferase (50 µM) was precipitated by adding 5 volumes of ice cold acetone and the obtained precipitate was dried at room temperature for 10 mins. The precipitated luciferase was resuspended in denaturation buffer (50 mM HEPES pH 7.5, 5 mM MgCl₂, 50 mM KCl, 6 M Guanidine HCl and 2 mM β-mercaptoethanol). The unfolded luciferase in denaturation buffer was exchanged with 1x HBS-EP+ (10 volumes) through 10 kDa centicon (Millipore) at 3200 rpm. Kindly note that unfolded luciferase sample should be processed immediately to avoid aggregation of luciferase. Unfolded luciferase was injected as analyte (0-1560 nM) for 3 minutes at flow rate of 30 µl/min. The single injection of 50 mM NaOH was used for regeneration. Bioevaluation software was used to process the sensograms. The $k_{on}$ (association rate) and $k_{off}$ (dissociation rate) were obtained through bivalent fitting. The second association rate constant ($kon_2$) is calculated through $kon_2$ (M$^{-1}$ s$^{-1}$) = $kon_2$ (RU$^{-1}$ s$^{-1}$) x Mr x 100 (where Mr is molecular weight). Molecular weight of PfHSP40 (88 kDa) and DNAJA1 (85 kDa) was determined through size exclusion chromatography by using protein standards (GE Healthcare 28-4038-42).

Small angle x-ray scattering (SAXS)

Purified PfHSP70-1 was incubated with 5 mM ADP and 100 µM NR-peptide, and concentrated through 3 kDa centicon (Millipore) upto 9 mg/mL in buffer (50 mM Tris pH 8.0, 5 mM MgCl₂, 500 mM KCl, 5 mM β-mercaptoethanol and 5 % glycerol) for SAXS experiment. Similarly, purified PfHSP40 was concentrated upto 9 mg/mL in buffer (50 mM Tris pH 8.0, 5 mM MgCl₂, 300 mM KCl, 1 mM β-mercaptoethanol and 5% glycerol) for SAXS experiment. The molecular weight of PfHSP70-1 and PfHSP40 was determined through size exclusion chromatography by using protein standards (GE Healthcare 28-4038-42). For PfHSP70-1 and PfHSP40 complex, equimolar concentration (26 µM) of
PfHSP70-1 and PfHSP40 were incubated with 5 mM ADP and 100 µM NR-peptide. The sample was concentrated up to 4 mg/mL. SAXS data was collected using Anton Paar SAXS space instrument at CSIR-Central Drug Research Institute (Lucknow, India) using a MYTHEN2 R 1K detector at a sample-detector distance of 0.3 m and at a wavelength of 0.154 nm. Scattering was measured at 10 °C. Two successive 1800 sec frames were collected. The data was normalized to the intensity of the transmitted beam and radially averaged. The scattering of the buffer was subtracted from solution scattering of samples. The data was processed by PRIMUSQT. The forward scattering intensity I(0) and radius of gyration R(g) were estimated using AUTOGNOM which was also used to evaluate the molecular size by plotting pair-distance distribution functions (PDDF). P(r) of scattering data is the representation in real space and reflects the particle’s shape. From SAXS scattering profile, 10 independent *ab initio* bead models were generated through DAMMIF. The average bead model was superimposed with homology models through SUPCOMB.

**Results**

*Plasmodium* harbors cytosolic, organellar (apicoplast, mitochondria) and exported HSP70-HSP40 machinery which exhibit stage-dependent differences in their expression [49, 50]. Amongst the plasmodial HSP70s, PfHSP70-1 is abundantly expressed in both asexual phase (in human) and sexual phase (in mosquito). PfHSP70-1 is essential for parasite survival and localized in both cytosol and nucleus [51]. Whereas, its paralog, PfHSP70-x is dispensable for its function, and is primarily expressed during ring stage of asexual erythrocytic phase in human (Supplementary Figure S1A). PfHSP70-x is exported into human RBC and interacts with exported type-II HSP40 [52, 53]. The co-chaperones of PfHSP70-1 include PfHSP40, PfSis1 and PfJ4 [40, 43, 54], of which PfHSP40 is the only abundantly expressed type-I cytosolic HSP40 (Supplementary Figure S1B). In the current study, structural-functional dynamics of cytosolic PfHSP70-1 and PfHSP40 machinery is probed. Like HSP70 family proteins, PfHSP70-1 harbors a N terminal nucleotide binding domain (NBD) and a C-terminal substrate binding domain (SBD). SBD comprises of β-barrel sheets (SBDβ) that extends into α-helical lid (SBDα) [55-57] (Scheme 1). Despite structural similarity between PfHSP70-1 and its human (HSPA1A) ortholog, they exhibit differences in their interaction interfaces (Supplementary Figure 2A). PfHSP70-1 interacts with PfHSP40 which contains a canonical N-terminal J-domain, glycine/phenylalanine (G/F) domain, two β-barrel domains for peptide/substrate binding, dimerization domain and a C-terminal tail [58-60] (Scheme 1). Sequence alignment of PfHSP40 and its human ortholog (DNAJA1) indicates significant divergence in their C-terminal region (Supplementary Figure 2B).
**Conformational dynamics of human and parasite chaperones**

We performed limited proteolysis of human and plasmodial chaperones (HSP70 and HSP40) to understand whether sequence diversity translates into differences in conformational dynamics. HSP70 is an allosteric chaperone that undergoes nucleotide-dependent conformational dynamics into an open state in presence of ATP and closed state in presence of ADP. HSP70 has high affinity towards substrate in ADP-bound state, whereas in ATP-bound state, HSP70 releases the substrate [55, 57, 61, 62]. In apo-state (without nucleotide or peptide), human HSP70 (HSPA1A) adopts ensemble conformations which are highly protease sensitive due to exposure of proteolytically labile regions to protease digestion (Figure 1).

However, in nucleotide-bound state (ADP or ATP), HSPA1A exhibits protease resistance [63]. The relative orientation of HSPA1A subdomains is different in ATP and ADP-bound state which give rise to subtle differences in protease digestion profile. In ATP-bound state, nucleotide binding domain (NBD) docks onto substrate binding domain (SBDβ) which intimately packs the inter-domain linker between these two domains and opens the cleft between SBDβ and SBDα (Docked or open conformation) [57]. While in the ADP-bound state, NBD is in undocked state with solvent-exposed inter-domain linker and SBDα is folded onto SBDβ (undocked or closed conformation). In presence of ADP and peptide, SBD has high affinity towards peptide which favors undocked or closed conformation and thus exposes protease sensitive regions. Interestingly, such dramatic differences in proteolytic profile were not observed for PfHSP70-1 in apo-state, nucleotide-bound state or in presence of peptide. This suggests that the relative orientation of subdomains, flexibility of inter-domain linker and loops affects the allosteric landscape of PfHSP70-1 (Figure 1). Similarly, limited proteolysis experiment was performed with human and parasite HSP40 in absence and presence of peptide. This HSP40-interacting peptide is derived from conserved C-terminal amino acid stretch (8-mer) of HSP70 [64, 65]. In this experiment, we observed that human HSP40 (DNAJA1) showed higher protease sensitivity in comparison to parasite HSP40 (PfHSP40) both in apo and peptide-bound state (Figure 2). Digestion profile obtained for DNAJA1 suggests that the likely cleavage site is in G/F region which serves as a flexible linker between the N-terminus J-domain and C-terminus peptide binding domain. In PfHSP40, the length and sequence of G/F region is different from DNAJA1 (Supplementary Figure S2B), which may contribute to differences in their conformational dynamics. We also performed CD melting studies to understand the thermal stability of human and parasite chaperones. Although, HSP70 and HSP40 orthologs had similar thermal stability, but they exhibited difference in their denaturation profile suggesting that they undergo distinct unfolding transitions (Supplementary Figure S3).
Differences in protein folding capacity of human and parasite chaperones

We wanted to compare the protein folding efficiency of human and plasmodial HSP70-40 chaperone pair. HSP40 stimulates ATPase activity of HSP70 by binding to its NBD and facilitates HSP70-mediated protein folding [66]. We observed that both human and plasmodial HSP40 stimulated the ATPase activity of their cognate HSP70 to a similar extent (Supplementary Figure S4 and Supplementary Table S2). We further probed into the foldase (refolding) and holdase (prevention of aggregation) activities of plasmodial and human HSP70-HSP40 at 25 °C. Plasmodial chaperones PfHSP70-1 and PfHSP40 exhibited better refolding of chemically denatured substrate than their human orthologs (HSPA1A and DNAJA1) (Figure 3A). In addition, plasmodial chaperones also exhibited better holdase activity for thermally denatured substrate (Figure 3B). Holdase activity suggest that plasmodial HSP40 (PfHSP40) has better ability to maintain the polypeptide in folding-competent state in comparison to its human ortholog (DNAJA1) (Figure 3B). PfHSP40 could also mediate a concentration-dependent increase in protein folding capacity, whereas, its human ortholog (DNAJA1) had moderate suppressive effect on protein folding yields at higher concentrations (Supplementary Figure S5). The foldase and holdase experiments were also performed at mildly denaturing temperature. At 37 °C, luciferase is vulnerable to misfolding and aggregation which resulted in low refolding yields for both plasmodial and human chaperones. Nevertheless, plasmodial chaperones exhibited better folding efficiency (especially holdase activity) than human chaperones at physiological temperature (Supplementary Figure S6). To confirm whether the observed better protein folding capacity of plasmodial chaperones is substrate-specific, we monitored refolding of heat denatured malate dehydrogenase (MDH). Plasmodial chaperones (PfHSP70-1 and PfHSP40) mediated better refolding of denatured MDH (Supplementary Figure S7). We also evaluated the oligomer remodeling ability of plasmodial and human chaperone pair by using limited proteolysis assay. Luciferase monomer is trypsin sensitive, whereas its oligomer is trypsin resistant. PfHSP70-1 and PfHSP40 system remodels the oligomer, making it sensitive to protease digestion (Figure 4A). This remodeling probably helps them to attain enzymatically active conformation resulting in concomitant restoration of luciferase activity (Figure 4B). Similarly, better disaggregation ability was observed for plasmodial chaperones (PfHSP70-1 and PfHSP40) over human chaperones (HSPA1A and DNAJA1) (Supplementary Figure S8).

Aforementioned biochemical observations suggest that PfHSP70-1 and PfHSP40 are better than their human orthologs. To identify the player which drives the folding efficiency, we swapped the chaperone pair in our biochemical experiments. Data indicates that PfHSP40 can efficiently cooperate with both plasmodial (PfHSP70-1) and human HSP70 (HSPA1A) to fold chemically and thermally denatured luciferase substrate (Figure 5). The better activity of PfHSP40 in comparison to DNAJA1 hints towards
functional differences in their binding to substrate and its transfer to HSP70. To gain insight into the kinetics of HSP40-substrate interaction, Surface Plasmon Resonance (SPR) experiments were performed with plasmodial or human HSP40 as ligand immobilized on the chip and unfolded luciferase as analyte. Sensograms were analyzed through various predefined fitting models, however satisfactory fitting results were obtained through bivalent interaction model (Figure 6). This involves two-step concerted binding, wherein the binding sites can have variable binding affinities [67] and this binding model is in agreement with the recent solution structure of type-I HSP40-unfolded substrate complex [38]. Kinetics data revealed that the association (k_{on1}) rate for substrate binding was moderately higher for PfHSP40, but dissociation (k_{off1}) rate was significantly lower than human ortholog (Table 1). k_{on1} and k_{off1} rates together contribute to the higher binding affinity (equilibrium association constant, K_{A1}) of PfHSP40 towards unfolded substrate in comparison to DNAJA1. The second binding site serves as low-binding affinity region and only modest difference in K_{A2} was observed for plasmodial and human HSP40. Overall, SPR data corroborates with refolding experiments that PfHSP40 efficiently binds to unfolded polypeptide and maintains it in folding-competent state to prevent its misfolding or aggregation.

**SAXS analysis of plasmodial HSP70, HSP40 and their complex**

We performed SAXS experiments to investigate the shape and conformational structure of PfHSP70-1, PfHSP40 and their complex. Under our experimental set-up, satisfactory solution scattering for plasmodial chaperones was obtained at high concentrations (7-9 mg/mL). For PfHSP70-1, we could not record solution scattering in apo and ATP-bound state due to concentration-dependent aggregation (at conc >5 mg/mL). Previous studies have also shown that HSP70 is prone to form dimers and multimers [68]. SAXS experiment was performed for PfHSP70-1 in presence of ADP and NR-peptide. The scattering profile was analyzed through Guinier plot to check the quality of sample. Guinier plot for PfHSP70-1 followed a linear profile suggesting the monodispersity of sample (Supplementary Figure S9). The calculated radius of gyration (R_g, 5.82 ± 0.30 nm) and maximum linear dimension (D_{max}, 16.56 nm) correlated with an extended monomer conformation. The bead model generated through DAMMIF fitted well with solution scattering (χ^2_{DAMMIF} 0.11). We used Ensemble Optimization Method (EOM) to generate structural model of PfHSP70-1 from experimental scattering (χ^2 0.84). The EOM-generated model was further analyzed through CRYSOL (χ^2 0.809). In presence of ADP and peptide, NBD of PfHSP70-1 was not docked onto SBD domain and SBDβ-SBDα cleft was in open state, probably due to dynamicity of α-helical lid (SBDα) (Figure 7) [63, 69]. Large V_{Porod}^L, R_g and D_{max} values obtained were probably due to dynamic rearrangement of domains of PfHSP70-1 or presence of heterogeneous populations in solution (undocked and partially docked) [63] (Table 2). In SAXS experiment with PfHSP40, the Guinier plot suggested that the protein sample is monodisperse and does not contain any
oligomeric species (Supplementary Figure S9). The radius of gyration (Rg: 5.06 ± 0.08 nm) and maximum linear dimension (D_{max} 15.0 nm) indicates a dimer conformation. Ab initio bead model generated through DAMMIF was fitted onto solution scattering ($\chi^2_{\text{DAMMIF}}$ 0.6). High resolution homology model (template: PDB 4J80) was also fitted onto solution scattering ($\chi^2_{\text{Crysol}}$ 2.613). The obtained SAXS data for PfHSP70-1 and PfHSP40 were deposited in SASBD bank under accession code of SASDHU5 and SASDHR5, respectively (Supplementary information). We also recorded solution scattering of PfHSP70-1 and PfHSP40 complex in presence of ADP and NR-peptide. However, the dynamic nature of interaction and inherent flexibility in their domains made the structural analysis of complex technically challenging. So far, full length HSP70-40 complex has not been determined, only the structures of the domains of prokaryotic or eukaryotic homologs have been solved through NMR and x-ray crystallography [38, 66, 70, 71]. In presence of ADP and peptide, the parasite chaperone complex comprises of PfHSP70-1 (monomer) and PfHSP40 (dimer) corresponding to molecular weight of 166 kDa (Table 2). DAMMIF was used to generate ab initio model, but the fitting onto solution scattering was not good due to poor buffer subtraction. We used ClusPro to build in silico model of PfHSP70-1 and PfHSP40 complex which was superimposed on the bead model generated through DAMMIF. The 3-D reconstruction with bead model gave hints into the overall architecture of the PfHSP70-1 and PfHSP40 complex.

Discussion

In this study, we highlight the fundamental differences in dynamics and chaperoning activity of malaria parasite chaperones (PfHSP70-1 and PfHSP40) and their human orthologs. Both PfHSP70-1 and its cytosolic co-chaperone (PfHSP40) are constitutively expressed during malaria parasite’s asexual cycle in human and sexual cycle in mosquito (Supplementary Figure S1A-B). These chaperones are also induced under stress conditions [40, 51], hence they participate both in house-keeping and stress-response related processes. PfHSP70-1 exhibits higher similarity to HSPA1A (inducible) over HSPA8 (constitutive) orthologs in human. Similarly, PfHSP40 has higher similarity with DNAJA1 amongst DNAJ protein family. Despite structural similarity, plasmodial and human HSP70-40 homologs exhibit striking differences in their conformational dynamics (Figure 1-2). The observed differences in conformational flexibility of PfHSP70-1 and HSPA1A suggest (i) differences in the number of exposed protease sensitive sites, (ii) variable length or flexibility of inter-domain linker and loops, and (iii) differences in the interaction interface of NBD-SBDβ and SBDβ-SBDα substrate-binding cleft. The conformational heterogeneity in docking/undocking of NBD-SBDβ and opening/closing of SBDβ-SBDα cleft dramatically affect the allosteric landscape of plasmodial and human HSP70 (Figure 1). A similar study showed allosteric differences between eukaryotic HSP70 (HSPA1A, HSC70) and the bacterial homolog
A recent study showed that mutations in the SBDβ-SBDα interface in HSPA1A affects the inter-domain communication between NBD and SBDβ [69]. In case of of plasmodial (PfHSP40) and human HSP40 (DNAJA1), variations in conformational dynamics may arise due to the differences in the flexibility of G/F region and relative orientation of domains (Figure 2).

Biochemical characterization reported in this work highlight that PfHSP70-1 and PfHSP40 machinery have better protein folding/refolding, aggregation inhibition, oligomer remodeling and disaggregate ability than their human orthologs (HSPA1A and DNAJA1) (Figures 3-5, Supplementary Figures S5-8). The physiological advantage of an efficient HSP70-40 system is associated with cost-benefit of refolding and disaggregation over an energetically-intensive process like re-synthesis of protein. The observed differences in HSP70-mediated protein folding in human and parasite is driven by their respective HSP40 co-chaperone. Sequence comparison of DNAJA1 and PfHSP40 shows that parasite HSP40 has diverged peptide binding domains hinting towards differences in their interaction with unfolded substrate (Supplementary Figure S2B). SPR experiment derived kinetic parameters explains why plasmodial HSP40 (PfHSP40) is better than its human ortholog. Higher association rate and slower dissociation rate allows PfHSP40 to bind unfolded substrate and maintain it in folding-competent state to minimize its misfolding or aggregation (Figure 3 and 6, Table 1). Our data suggests that the functional diversity of PfHSP40 (type-I cytosolic HSP40) gives an edge over its human ortholog (DNAJA1) in driving HSP70-mediated protein folding (Figure 3-4, Supplementary Figure S5-8). Infact, PfHSP40 could also efficiently coordinate with human HSP70 for folding of client-substrate (Figure 5). Using yeast-2-hybrid and pull-down experiments, other plasmodial HSP40s (especially type-II HSP40) have also been shown to interact with human HSP70 [72].

The functional diversity of HSP40 interaction with HSP70 and substrate is influenced by the sequence variation of in its peptide binding domains [37, 38, 73]. For instance, yeast HSP40s (Ydj1 and Sis1) are suggested to have differences in binding to HSP70 and client-substrate. Ydj1 can engage both client-substrate and HSP70, whereas, Sis1 cannot interact with them simultaneously due to overlapping binding sites [38]. Similarly human cytosolic type-I HSP40s (DNAJA1 and DNAJA2) show distinct differences in substrate binding and release [37]. At high concentrations, DNAJA1 impairs the protein refolding yields due to competing reactions for binding to HSP70 and substrate [67]. On similar lines, we observed that high concentration of DNAJA1 did not enhance the protein refolding yields and rather had modest suppressive effect. Whereas, its plasmodial homolog enhanced protein folding yields in concentration-dependent manner (Supplementary Figure S5).
We also probed into the structural architecture of parasite chaperones (PfHSP70-1, PfHSP40 and their complex). Till date, high resolution structures are only available for domains of prokaryotic and eukaryotic HSP70 and HSP40 homologs [60-62, 74, 75]; and not for their full length proteins. Conformational dynamics and transient nature of chaperone and co-chaperone interaction makes structural characterization of HSP70-HSP40 complex technically challenging. Using SAXS, we obtained insights into the structural properties of PfHSP70-1, PfHSP40 and their complex. SAXS data for PfHSP70-1 shows that it adopts an extended conformation in presence of ADP and peptide (Figure 7). This correlates with SAXS data of *Leishmania* mitochondrial HSP70 [76]. Similarly, solution scattering for PfHSP40 indicates that it exist as a dimer as reported previously for bacterial HSP40 [77]. We also collected SAXS data for PfHSP70-1-40 complex. Although, the bead model generated gave poor fitting to solution scattering, nevertheless, it gave hints into the overall architecture of complex comprising of PfHSP70-1 and PfHSP40 dimer. Despite sequence variations, we observe an evolutionary conserved electrostatic interaction between J-domain of PfHSP40 and NBD of PfHSP70-1 which correlates with the previous data for bacterial DnaK-DnaJ complex (Figure 7). This interaction could be driven by helix II in J-domain and IIA lobe in NBD, as observed in case of DnaK-DnaJ complex [70]. MD simulation study on DnaK-DnaJ complex suggest that conformational flexibility of J-domain facilitates additional interactions with DnaK at inter-domain linker and SBDβ [78]. Similar MD study with PfHSP70-x and exported type-II HSP40 (PFA0660w) shows that J-domain makes stable interactions with NBD and SBD of PfHSP70-x [79] which corroborates with recent structural analysis of complex formed by NBD of PfHSP70-x and J domain of PFA0660w [80]. This MD study also predicted an additional interaction site wherein, the G/F region of PFA0660w makes contact with SBDβ of PfHSP70-x [79]. A recent smFRET study for human HSP70 (hHSP70) and Hdj1 (type-II HSP40) chaperone pair suggests that Hdj1 dimer induces dimerization of hHSP70 to form a hetero-tetramer complex [81]. Transient dimerization of HSP70 for efficient interaction with HSP40 is also reported for bacterial homologs [82]. A NMR based structural characterization of interaction between bacterial HSP40 (ttHSP40) and C-terminal tail of ttHSP70 (15 amino acids long polypeptide) shows that this C-terminal tail makes contact with peptide binding domain II of ttHSP40. However, this interaction interface varies in different eukaryotic HSP40s [38]. Numerous literature observations across species suggest that the conformational heterogeneity of HSP70-HSP40 complex depends upon nucleotide, co-chaperone and substrate [38, 66, 70, 71], leading to functional versatility in their biological roles.

Our biochemical data give hints into how the cytosolic HSP70-40 machinery is chaperoning parasite’s metastable proteome against the proteotoxic stress conditions encountered during lifecycle in cold-blooded mosquito and warm-blooded human host. Similar investigations are required to understand
distinct chaperoning capabilities of organellar and exported plasmodial chaperones. Studies have shown that exported chaperones are involved in trafficking of parasite cargo proteins (in unfolded or partially folded state) into cytosol of human RBCs, where they refold with help of human chaperones and/or exported parasite chaperones [83-85]. The transient organelles like maurer’s cleft, J-dots, formed in human RBCs upon *P. falciparum* infection, harbor exported HSP70-40 which mediate the trafficking and assembly of parasite virulence factor on RBC membrane [29, 84, 86]. This facilitates remodeling of RBC membrane, increases its cytoadherence and prevents the clearance of infected-RBCs by spleen [87]. Human RBCs are e-nucleated and transcriptionally/translationally silent, thus they cannot mount any cellular response to upregulate their chaperones during *Plasmodium* invasion into RBCs. Whereas, parasite reprograms its cellular machinery and has specific upregulation of its HSPs during infection and febrile episodes [88]. Further, the cellular abundance of parasite’s protein folding machinery during infection and disease severity is influenced by the growth rate or metabolic state of parasite and host immune response [89, 90]. Therefore, a systematic molecular investigation of parasite’s protein folding machinery will provide insights into their versatile roles during progression of infection. And comprehensive biochemical characterization of components of this machinery will highlight their unique allosteric landscapes and interaction-kinetics which can further open avenue for design of small-molecules/peptides selective for parasite chaperones.

**Author Contributions**

MA and NK designed the study. NK coordinated and supervised the study. MA, PN and LSK performed cloning and standardized protein purifications. MA, AT, VK, CP performed protein purifications, biochemical assays, CD melting and generated polyclonal antibody. SKS gave inputs for biochemical experiments. MA and AS performed SPR experiments, AS performed SAXS experiments under supervision of RR. MA and NK wrote manuscript with inputs from all authors.

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Figure legends

Scheme 1. Homology model of plasmodial HSP70 (PfHSP70-1) and HSP40 (PfHSP40) chaperones built through Phyre2. PfHSP70-1 model was generated in open-state (5E84) [ref 57], wherein the substrate binding domain (β-SBD and α-helical lid) docked onto the nucleotide binding domain (NBD). PfHSP40 model was built using 4J80 as template [ref 60]. Type-I HSP40 comprises of J-domain, G/F region, peptide binding domains (I and II), dimerization domain (DD) and C-terminal amino acid stretch. The peptide binding domain-1 contains zinc-finger like regions which comprises of CXXCXGXRG motifs (marked in black).

Figure 1. Probing conformational dynamics of human and parasite HSP70. (A) Schematic representation of docked and undocked state of HSP70. Color code for different domains is: NBD (magenta), inter-domain linker (black), β-SBD (green) and α-helical lid (yellow) Model built through Phyre2 using templates (2KHO for undocked and 4B9Q for docked-state, ref: 61-62). (B) Limited proteolysis assay for HSPA1A (40 µM) and PfHSP70-1 (40 µM) using trypsin at 25 °C in apo-state (without nucleotide and peptide), 5 mM ADP/ATP bound state in absence or presence of NR-peptide (120 µM). The samples were run on SDS-PAGE and analyzed through Commassie staining. Gel represents the protease digestion profile for full length human and plasmodial HSP70 orthologs. A faint degradation band is observed in purified PfHSP70-1.

Figure 2. Probing into conformational flexibility of human and plasmodial HSP40. Limited proteolysis assay for DNAJA1 (40 µM) and PfHSP40 (40 µM) using trypsin at 25 °C in apo-state (without nucleotide and peptide) and presence of ter-70 peptide (120 µM). The samples were run on SDS-PAGE and analyzed through Commassie staining.

Figure 3. Assessing the differences in protein folding efficiency of human and plasmodial HSP70-HSP40 chaperone pair. (A) Foldase activity was determined through refolding of chemically (6 M GdHCl) denatured luciferase (0.3 µM) in the absence and presence of human and plasmodial orthologs (1 µM HSP70 and 4 µM HSP40) at 25 °C. (B) Holdase activity was determined through thermal denaturation of luciferase (0.3 µM, 42 °C for 10 min) in the absence and presence of human and plasmodial HSP40 (4 µM DNAJA1 or PfHSP40), followed by refolding at 25 °C in presence of respective HSP70 (1 µM HSPA1A or PfHSP70-1). Data is represented as % luciferase activity obtained after normalization with luminescence values of natively folded luciferase. Data represents mean±SD, n=3.

Figure 4. Comparison of oligomer remodeling activity of human and plasmodial orthologs. (a) Limited proteolysis experiment was performed for luciferase monomer (4 µg) and oligomer (4 µg). Luciferase monomer and oligomer were incubated with or without plasmodial and human chaperones (1 µM HSP70 and 4 µM HSP40) for 30 min at 25 °C, followed by trypsin digestion.
at 25 °C. The samples were analyzed through western blotting with luciferase antibody. (b) Luciferase activity of oligomer (4 µg) was monitored at different time-points after incubation with plasmodial and human chaperones (1 µM HSP70 and 4 µM HSP40) at 25 °C. Data represents mean±SD, n=3.

**Figure 5.** Protein folding ability of swapped chaperone pair. Foldase and holdase activities were assessed for chemically (6 M GdHCl) and thermally (42 °C for 10 mins) denatured luciferase (0.3 µM). Luminescence was recorded for denatured luciferase in presence of plasmodial/human orthologs and their swapped chaperone-pair (1 µM HSP70 and 4 µM HSP40 after 30 min incubation at 25 °C). Data is represented as % luciferase activity obtained after normalization with luminescence values of natively folded luciferase. Data represents mean±SD, n=3.

**Figure 6.** Determining the kinetics of HSP40-substrate interaction. DNAJA1 or PfHSP40 (250 nM) was immobilized on CM5 sensor chip. (A and B) Sensograms for HSP40 (DNAJA1 and PfHSP40) interaction with increasing concentration of unfolded substrate (luciferase, 0-1560 nM). The data was fitted with bivalent binding mode (red lines). The residual plots (bottom panel) represent the deviation of experimental binding data from their respective fitted curves.

**Figure 7.** SAXS analysis of PfHSP70-1, PfHSP40 and their complex. Superimposition of homology models of PfHSP70-1 and PfHSP40 onto the ab initio dummy-atom envelope constructed through DAMMIF from experimental small angle x-ray scattering. SASBDB-IDs are SASDHU5 (PfHSP70-1) and SASDHR5 (PfHSP40). The samples for solution scattering of PfHSP70-1 and its complex with PfHSP40 was prepared in presence of ADP and NR-peptide.
A) docked/open conformation

B) HSPA1A

- 0 5 10 15 (min)
- Apo
- ADP
- ATP
- ADP + peptide
- ATP + peptide

PfHSP70-1

- 0 5 10 15 (min)
- Apo
- ADP
- ATP
- ADP + peptide
- ATP + peptide
Foldase activity
Luciferase → Chemical denaturation 6M GdHCl → Refolding by human or plasmodial chaperones

(A) Human
- No chaperones
- DNAJA1 alone
- HSPA1A alone
- HSPA1A & DNAJA1

Plasmodium
- No chaperones
- PfHSP40 alone
- PfHSP70-1 alone
- PfHSP70-1 & PfHSP40

(B) Holdase activity
Luciferase → Thermal denaturation 42 °C, 10 min (DNAJA1 or PfHSP40) → Refolding in presence of human or plasmodial HSP70

(A) Human
- No chaperones
- DNAJA1 alone
- HSPA1A alone
- HSPA1A & DNAJA1

Plasmodium
- No chaperones
- PfHSP40 alone
- PfHSP70-1 alone
- PfHSP70-1 & PfHSP40
Table 1. Kinetic constants for interaction of human and plasmodial HSP40 with unfolded substrate.

| HSP40   | $k_{on1}$ (M$^{-1}$s$^{-1}$) | $k_{off1}$ (s$^{-1}$) | $K_A1$ | $k_{on2}$ (M$^{-1}$s$^{-1}$) | $k_{off2}$ (s$^{-1}$) | $K_A2$ |
|---------|----------------------------|-----------------------|--------|-----------------------------|-----------------------|--------|
| DNAJA1  | 3.57×10$^3$                | 9.56×10$^{-4}$        | 3.73×10$^6$ | 71.31                      | 1.35×10$^{-1}$        | 5.28×10$^2$ |
| PfHSP40 | 9.89×10$^3$                | 1.14×10$^{-4}$        | 8.68×10$^7$ | 7.95                       | 7.29×10$^{-2}$        | 1.09×10$^2$ |

Kinetic constants were determined through SPR experiments. Human HSP40 (DNAJA1) and plasmodial HSP40 (PfHSP40) were used as ligand and unfolded luciferase was used as analyte. Sensograms were fitted with bivalent fitting to obtain rate constants. Equilibrium association constant ($K_A$) is the ratio of $k_{on}/k_{off}$. 
Table 2. SAXS analysis of plasmodial HSP40, HSP70 and their complex

| SASBDB ID       | PfHSP70-1       | PfHSP40       | PfHSP70-1 and PfHSP40 complex |
|-----------------|-----------------|---------------|-------------------------------|
| Structural parameters | SASDHU5 | SASDHR5 | -                           |
| Theoretical MW  | 73.9 kDa        | 97.0 kDa      | 170.9 kDa                     |
| Experimental MW | 74.8 kDa        | 88.0 kDa      | 166.0 kDa                     |
| Stokes radius (nm) | 1.87   | 1.94         | 2.22                          |
| c I(0) [from Guinier] | 113100 | 77410        | 55860                         |
| d ) Rg (nm) [from Guinier] | 5.82 ± 0.30 | 5.06 ± 0.08 | 5.99 ± 3.26                   |
| d I(0) [from P(r)] | 113100 | 77410        | 55860                         |
| Rg (nm) [from P(r)] | 5.81   | 5.06         | 5.99                          |
| Dmax (nm)       | 16.56           | 15.0          | 17.34                         |
| e Vporod (nm³)  | 450             | 339           | -                             |
| Modeling parameters |       |               |                               |
| Chi² (EOM)      | 0.84            | -             | -                             |
| f Chi² (CRYSOLO) | 0.809           | 2.61          | -                             |
| g Chi² (DAMMIF) | 0.11            | 0.6           | 0.006                         |
| h NSD           | 3.92            | 3.0           | 4.07                          |

- Experimental MW determined through SEC (S200 increase 10/300); b Stokes radius is the hydrodynamic radius determined through SEC. c,d I(0) is the scattering intensity of sample; Rg is radius of gyration; Dmax is maximum linear dimension; e Vporod is the volume of scattering particle obtained through Porod-Debye law; f Fitting of solution scattering of macromolecules with known atomic structure into experimental scattering obtained through SAXS; g ab initio shape determination. Due to poor buffer match, DAMMIF value for complex is very low; h Fitting of high resolution model on experimental scattering to obtain normalized spatial discrepancy (NSD). NSD value is obtained from SUPCOMB. The obtained NSD is >1 because high resolution data is not available for plasmodial HSPs. Homology structures are generated using PDB structures of bacterial orthologs.