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Hsp70-1 from *Plasmodium falciparum*: Protein stability, domain analysis and chaperone activity

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ABSTRACT

P. falciparum contains six copies of the *Hsp70* gene of which PfHsp70-1 is important in the parasite's lifecycle. The protein consists of two domains like other Hsp70s but has an unusually long C-terminal tail. The full-length protein is stable towards high temperatures and chemical denaturants. Fluorescence and circular dichroism studies demonstrate that the ~42 kDa N-terminal/nucleotide-binding domain (NBD) is relatively unstable in isolation. Addition of the ~35 kDa C-terminal domain with an extended tail containing an EEVD motif confers thermal stability and makes it less susceptible to thermal denaturation. This suggests that the C-terminal domain functions as a stabilization domain. PfHsp70-1 possesses a chaperone activity in addition to other functions reported earlier. We report that the chaperone activity of PfHsp70-1 is enhanced in the presence of *P. falciparum* Hsp40 (Pfj1, PFD0465w), the homolog of bacterial DnaJ. The present work represents the first evidence for functional interactions between the PfHsp70-1 and Pfj1.

1. Introduction

Plasmodium falciparum accounts for the highest mortality rate among the four species of protozoan parasites known to cause malaria [1]. The lifecycle of malarial parasite is complex and involves two hosts *viz*. the cold-blooded mosquito vector and the warm blooded human host. It has therefore to adapt to a range of temperatures and physiological changes [2]. Febrile episodes which are a characteristic of malaria, involves an increase in the body temperature to about 41 °C. Increase in the release of certain cytokines is implicated in the symptoms [3]. It has been suggested that enhanced production of chaperones (Hsps) is a survival strategy applied by the parasite to face temperature variations [2]. They are also important in pathogenic organisms for host cell invasions and related events [4,5].

A recent analysis of the chaperone network existing in *P. falciparum* suggests that Hsps are important for the parasite's adaptation to the host [6]. Their accelerated synthesis is the organism's way of combating stress conditions. Some Hsps like PfHsp90 are being recognized as novel drug targets while chaperones of the Hsp40 family are suggested to be involved in chaperone-mediated translocation, folding, assembly and regulation of host and parasite proteins [7]. Other chaperones like PfHsp70 are considered to be novel potential vaccine candidates [8]. The 70-kDa Hsp70 family has been characterized and is relatively abundant in various cellular compartments [9]. Structurally, Hsp70 proteins consist mainly of an N-terminal nucleotide-binding domain (NBD) that is attached to the substrate-binding domain (SBD) through a linker region that is involved in allosteric regulation of NBD and SBD activities [10,11]. *P. falciparum* harbors at least six *Hsp70* genes which possess individually specific localization signals [12]. PfHsp70-1, a cytosolic protein and more recently PfHsp70-2, a protein localized in the endoplasmic reticulum, have been cloned and purified by at least two groups [13,14]. These two proteins differ in an EEVD motif that occurs only in PfHsp70-1 and enables it to bind to co-chaperones [14].

PfHsp70-1 has been shown to be ~74 kDa and is expressed in the blood stage of the parasite. It has been implicated in the transport of nuclear encoded proteins destined to the apicoplast [15]. A modelling study has predicted that PfHsp70-1 contains conserved Hsp40 binding residues that are spatially distributed in the vicinity of the inter-domain interface of the protein [11]. It has also been shown to possess chaperone activities *in vitro* that are enhanced in the presence of ATP [14]. Recombinant PfHsp70-1 has also been able to suppress the thermosensitivity of an *E. coli* DnaK mutant strain [16] suggesting that its chaperone activities might be similar to that of bacterial counterparts [17]. However, no reports with associated co-chaperones like: Hsp40 (DnaJ) analogous to that reported in bacterial Hsp70 homologs are known. *P. falciparum* harbors ~43 Hsp40 homologs which have been cloned and characterized [18]. Several of them, especially those containing a PEXEL/HT (*plasmodium* export element/host targeting signal) are unique to the parasite [7]. It has also been suggested that

Abbreviations: CD, circular dichroism; GdmCl, guanidium chloride; G6PDH, Glucose-6-phosphate dehydrogenase; NBD, nucleotide-binding domain; P-Ctr, substrate-binding domain with C-terminal; *Pfhsp70-1*, *Plasmodium falciparum* heat shock protein 70-1; SBD, substrate-binding domain.

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the specificity of the association of Hsp70 protein to a particular Hsp40 protein partner is governed by the signature J-domain of the latter. More specifically, a domain swapping study established that a helix II and HPD motifs along with some conserved residues of the second and third helix constituting the J-domain play an important role in giving fidelity and specificity to Hsp40 and Hsp70 interactions. In the majority of the studies which includes mutational analysis of residues involving the J-domain of Pfj1, it was found that constructs possessing an intact HPD motif retained functionality [19]. We therefore used Pfj1 (61–672 amino-acid residues), a type I Hsp40 [7] which contains the necessary domains for the chaperone assays.

Sequence wise PfHsp70-1 has a GGMP motif which is specific to Hsp70 from parasitic protozoans and might play a role in the immune response of the host [13]. Recently Hsps are drawing attention as novel therapeutic targets. A focus is on the development of compounds that efficiently work as chaperone modulators; as inhibitors for chaperones like Hsp90 and inducers for chaperones like Hsp70. These exhibit high efficacy in case of recovery from diseases including ischemic heart disease, diabetes and neuro degeneration [20]. Hsp70 has been implicated as a therapeutic target in Parkinson's disease where it is active in degrading aberrant proteins. Its anti-apoptotic activity has also been cited to be significant for the role it plays in neuro protection [21]. The possibility of designing novel anti-malarials that interfere with the Hsp70 interactions is attracting attention [12]. An inhibitor of PfHsp70-1, 15-Deoxyspergualin has been shown to modulate its function by binding to the EEVD motif at the C-terminal [14]. Many smaller compounds similar to 15-Deoxyspergualin and NSC 630668-R/1, that either affects the Hsp70 ATPase activity or the Hsp70–Hsp40 interactions are being explored. The studies also include compounds that specifically target Hsp40 co-chaperones without affecting the intrinsic activity of Hsp70 [22]. One such modulator *viz.* (−)-epigallocatechin-3-gallate, has recently been shown to bind to the ATPase domain of Hsp70 promoting apoptosis in colorectal cancer [23]. Addition of the C-terminal fragment of the *Hsp70* gene has also reportedly led to an enhancement in the potency of a DNA vaccine [24]. Obviously, these efforts need to be supplemented by a more detailed characterization of the individual proteins and the roles of individual domains in protein function and stability.

In the present report, we have cloned and purified PfHsp70-1 with an aim to understand its stability properties. We have also purified various domains *viz.* an N-terminal ATPase domain, a substrate-binding domain and the latter with an extended C-terminal tail (P-Ctr) in order to characterize them and understand their roles in structure and stability. It has been conjectured that PfHsp70-1 might interact with some of the parasite Hsp40 homologs (DnaJ in bacteria) although the identity of the actual interacting proteins is still under speculation. We have demonstrated a functional interaction between PfHsp70-1 and an Hsp40 (Pfj1, PFD0465w) that results in an enhancement of the chaperone activity of PfHsp70-1, analogous to bacteria. PfHsp70-1 retains its ATPase activity at high temperatures (80 °C) and also in the presence of chemical denaturants. As reported earlier, the protein exhibits a basal chaperone activity in the absence of ATP that is enhanced in the presence of the nucleotide. In view of the importance of the activity of the protein at extreme conditions necessary for the parasite's survival, we have dissected the roles of the individual domains in protein stability through circular dichroism and fluorescence studies. The thermal and chemical denaturation studies with the full-length and truncated variants show that the protein's C-terminal domain consisting of the SBD and the elongated C-terminal segment is highly stable. It interacts extensively with the N-terminal nucleotide-binding domain to confer additional stability on the latter domain. Overall, this is the first report involving the biophysical characterization of the individual domains of PfHsp70-1 and the results support the strategy of disrupting the interactions of the C-terminal tail of the protein for the development of novel anti-malarials.

2. Materials and methods

2.1. Bioinformatics analysis, fold prediction and domain organization

The complete protein sequence (accession no: PF08_0054) was retrieved from PlasmoDB (http://plasmodb.org/plasmo/). Homology searches were performed using BLASTP (http://www.ncbi.nlm.nih.gov/blast/BLAST.cgi) against the PDB (http://www.rcsb.org/). The fold prediction analysis was carried out using the 3DPSSM server (http://www.sbg.bio.ic.ac.uk/3dpssm/index2.html).

2.2. Cloning, over-expression and purification

2.2.1. Full-length Hsp70-1

The *Hsp70-1* gene was PCR amplified from *P. falciparum* genomic DNA using the primers: sense primer 1. 5′-GC**GGATCCATGG**CTA-GTGCAAAAGGTTC-3′ and anti-sense primer 5′-CG**AAGCTT**TTAAT-CAACTTCTTCAACTGT-3′ containing BamHI and NcoI restriction sites in sense primer and Hind III restriction site in anti-sense primer. The PCR product (2.034 kb) was subsequently cloned into the T7-based expression plasmid pET 21d (Novagen) at the NcoI and Hind III sites. The resulting construct which contains a C-terminal hexa-histidine tag was co-transformed with the RIG plasmid [25] into *E. coli* C41 (DE3) and grown in LB medium. 1 mM isopropyl-1-thio-β-D-galactopyranoside was added at an OD_{600} of ~0.8 to induce protein expression. A Ni^{2+}-IDA column (M/s GE Biosciences), along with a linear gradient of imidazole (10–500 mM) in a buffer containing 50 mM Tris–Cl, pH 8.0 and 50 mM NaCl was used to purify the protein. The protein eluted between 275 and 375 mM imidazole concentrations. It was subsequently concentrated using a centricon (M/s Millipore, USA) and incubated in 8 M urea buffer (50 mM Tris, 50 mM NaCl, 5 mM EDTA, 8 M urea) at 4 °C. Initial purification trials involving affinity purification and ion exchange chromatographic techniques yielded only partially purified PfHsp70-1. We therefore resorted to the 8 M urea incubation step which resulted in the enhanced purification of PfHsp70-1. The protein was further subjected to gel filtration chromatography using a Superdex 200 HR 10/30 column mounted on AKTA FPLC system (M/s GE Biosciences). The column was pre-equilibrated with the urea buffer. The column was calibrated using standard molecular weight markers (M/s GE Biosciences). The relative elution volume was calculated as:

$$Kav = Ve - Vo / Vg - Vo \quad (1)$$

where, Ve is the elution volume, Vo is the void volume as determined by the elution profile of Blue Dextran (2000 kDa) and Vg is the geometric column volume.

PfHsp70-1 elutes as a higher oligomer. Purified fractions were extensively dialyzed against buffer containing 50 mM Tris pH: 8.0, 50 mM NaCl, 5 mM EDTA and then concentrated using a 50 kDa centricon (M/s Millipore, USA). which was subsequently used for ATPase and chaperone assays.

2.2.2. ATPase domain (NBD)

The ATPase domain fragment was PCR amplified from *P. falciparum* genomic DNA using the primers: sense primer 1. 5′-GCA**CCATGG**AATC-GAATATCGCTATTGGAATT-3′ and anti-sense primer 5′-TAT**GGATCCAA-GCTT**ACCAGATAAAATGGCTGCATGTAC-3′ containing NcoI restriction site in sense primer and BamHI and Hind III restriction sites in anti-sense primer. The PCR product (1.04 kb) was subsequently cloned into T7-based expression plasmid pET 21d (Novagen) at the same sites. pET-21d carrying the ATPase gene was co transformed with RIG plasmid [25] into *E. coli* C41(DE3) and grown in LB medium at 37 °C initially and 30 °C after induction for 10 h. Over-expression was achieved using 0.5 mM isopropyl-1-thio-β-D-galactopyranoside added at an OD_{600} of ~0.6 in the medium. A Ni^{2+}-IDA column using

a linear gradient of imidazole (10–500 mM) in a buffer containing 50 mM Tris–Cl, pH 8.0 and 300 mM NaCl was used and protein eluted at 235–375 mM imidazole concentration. The eluted fractions were concentrated using centricon and finally purified through gel filtration chromatography.

2.2.3. Substrate-binding domain (SBD)

The substrate-binding domain was cloned into T5-based expression plasmid pQE60 (Qiagen) using primers 5′-CATG**CCATGG**CAATTAACCCAGATGAAGCTGTT-3′ as sense and 5′-ACG**GGATCCAAGCTT**TTCAAAGCTGTTTCTTGC-3′ as anti-sense primer containing NcoI and BamHI restriction sites respectively. The construct co transformed with RIG [25] in *E. coli* XL1Blue cells, over expressed as C-terminal his-tagged protein in TB medium at 30 °C, when induced with 0.3 mM IPTG at OD_{600} of ~0.5. Protein was purified using Ni^{2+}-IDA affinity chromatography, using buffers containing 50 mM Tris–Cl, pH 8.0 and 300 mM NaCl, with a linear gradient (10–500 mM) of imidazole. Protein eluted around 175–275 mM imidazole was precipitated using 60% ammonium sulphate and dissolved in minimum volume of gel filtration running buffer (50 mM Tris–Cl, pH8.0, 50 mM NaCl, 5 mM EDTA). Final step involved gel filtration chromatography using S-200 column (*M/s GE Biosciences*).

2.2.4. P-Ctr

The substrate-binding domain along with the C-terminal (P-Ctr) was cloned into pQE60 with C-terminal His-tag using sense primer 5′-CATG**CCATGG**CAATTAACCCAGATGAAGCTGTT-3′, and anti-sense primer 5′-CCC**AAGCTTGGATCC**ATCAACTTCTTCAACTGTTG-3′ containing NcoI restriction site in sense primer and Hind III, BamHI restriction sites in the anti-sense primer. The 0.942 kb construct was co transformed with RIG [25] in *E. coli* XL1Blue cells. Over-expression was achieved in YT medium by inducing with 0.4 mM of IPTG at an A_{600} of 0.7 and grown for 16 h at 30 °C. Protein was purified using Ni^{2+}-IDA affinity chromatography with binding buffer containing 50 mM Tris–HCl pH 7.0, 300 mM NaCl, 10 mM imidazole. The column was washed extensively with the same buffer. The protein was eluted using a linear gradient of buffer B containing 700 mM imidazole. The protein eluted at 35–55% imidazole (235–375 mM). Purity was monitored by electrophoresing the samples on a 12% SDS-PAGE. Fractions with less contamination were pooled and precipitated with 60% ammonium sulphate. Precipitate was dissolved in minimum volume of gel filtration (50 mM Tris–Cl, pH 7.0, 50 mM NaCl, 1 mM EDTA) and trace contaminants were removed by gel filtration on a Superdex S-200 HR10/30 column mounted on AKTA FPLC system.

2.3. Chaperone assay

The enzymatic activity of Glucose-6-phosphate dehydrogenase (G6PDH) and alpha glucosidase used as substrates in the assay was measured using spectroscopic methods as reported previously [17,26]. PfHsp70-1 mediated reactivation assays were carried at 25 °C in buffer containing 50 mM Tris pH: 7.5, 20 mM KCl, 10 mM $MgCl_2$ supplemented by 4 mM ATP and an ATP-regeneration system (5 mM phosphoenol pyruvate and 20 ng/ml pyruvate kinase). In brief, G6PDH (90 nM) from *L. mesentroides* (Sigma) and alpha glucosidase (0.15 μM) from *B. stearothermophilus* (Sigma) were heat denatured at 40 °C for 20 min. The denatured enzymes were then incubated with PfHsp70-1 alone and PfHsp70-1 along with co-chaperone Pfj1 (in the ratio 5:1), supplementing the reaction mix with ATP-regeneration system as mentioned above. The Hsp40 (Pfj1) pQE30 based expression vector used in the present experiments is a kind gift from Dr. Saman Habib, MSB division, *Central Drug Research Institute*, Lucknow. It encodes for residues 61–672 which corresponds to the mature protein and contains an N-terminal hexa-histidine tag. The protein was purified routinely by affinity chromatography using a Ni^{2+}-IDA column (M/s GE Biosciences). The protein was dialyzed against a buffer containing (50 mM Tris pH: 8.0, 100 mM NaCl, 3 mM EDTA, 20% Glycerol) for the experiments. All protein concentrations are expressed in protomers, regardless of the oligomeric status of the proteins. The intrinsic chaperone activity of Pfj1 alone was tested but no chaperone activity was observed in the absence of PfHsp70-1.

2.4. Nucleotide hydrolysis assay

A malachite green based ATPase assay [27] was used to measure ATP hydrolysis by PfHsp70-1 with some modifications. The reaction mixture contained a fixed concentration (5 μM) of PfHsp70-1, ATP concentration varying from 10 μM to 900 μM and 2 μl of 10× reaction buffer (500 mM Tris,pH: 7.5, 200 mM KCl, 200 mM $MgCl_2$) making the total volume 20 μl. The samples were incubated at 37 °C for 30 min. The reaction was stopped by adding 180 μl of freshly prepared malachite green–ammonium molybdate reagent. This reagent contained 3 volumes of 0.045% (w/v) malachite green hydrochloride (Sigma), 1 volume of ammonium molybdate (4.2% (w/v) in 4 N HCl), and 1/50 volume of 1% (w/v) Triton X-100. After 1 min at room temperature, 100 μl of 34% (w/v) citric acid was added to stop color development. The samples were kept at room temperature and measured photometrically in 96-well plate at a fixed wavelength of 630 nm. To quantitate the amounts of enzymatically released P_i, the samples (in triplicates) were compared with a standard curve, which was prepared with dilutions of a standard solution (1 mM KH_2PO_4 in 0.01 N H_2SO_4) over a range of 10 to 250 nM phosphate.

PfHsp70-1 was scanned for its ATPase activity at different temperatures and in the presence of different denaturant concentrations. The procedure followed was similar to above only the incubation temperatures were different. For activity in the presence of denaturants, the buffer was supplemented with the required amount of denaturant concentration. The ATP hydrolysis values were corrected for background by subtracting the value obtained for a reaction mixture containing no protein.

2.4.1. Radioactivity based ATPase assay

ATP hydrolysis activity of NBD (1 μg) was assayed by analyzing the release of [28] Pi in 50 mM Tris–Cl buffer pH7.5, 20 mM KCl, 10mM$MgCl_2$ using 1 μCi [γ-^{32}P]ATP at 37 °C. Initially protein concentration was varied from 0.5 μg–4 μg. The time dependent steady state ATPase activity was measured using 1 μg protein. Samples were drawn at an interval of 15 min till 2 h The reaction was terminated by 0.75 M NaH_2PO4 (pH 3.65), spotted onto a silica gel thin-layer-chromatography plate, developed in 1 M LiCl and 0.35 M NaH_2PO4 (pH 3.65) and autoradiographed [29]. Densitometry analysis was done with Image QuantTL software (GE Biosciences). Background values (without protein) were subtracted. The activity was expressed as % activity (%ATP hydrolysed).

$$\%\,\text{Activity} = (I_{\text{Pi}} / I_{\text{Total}})*100. \quad (2)$$

I_{Pi}, intensity of the inorganic phosphate band (fast moving band on TLC) and I_{Total}, total intensity *i.e.*, unhydrolyzed ATP and the released inorganic phosphate.Cold ATP was not used in any of the reactions.

2.5. CD spectroscopy

CD measurements were made with a Jasco J800 spectropolarimeter with a Peltier type cell holder that allows for temperature control. The spectra were calculated from 1 μM protein samples dissolved in a 20 mM Tris–HCl pH8.0 and 30 mM NaCl buffer. For CD studies with nucleotide binding, the samples were prepared as above with the change that nucleotides at the required concentrations were added into the reaction mixtures. Typical spectra were recorded from 190 to 250 nm for far UV at a scan speed of 10 nm per min, with each spectrum representing an average of 3 accumulations. Each spectrum

was an average of two scans. During acquisition the samples were maintained at a regulated temperature (25 ± 5 °C). The results are expressed as molar ellitipicity, $[\theta]$, or the residue ellipticity, $[\theta]_{MRW}$, are calculated from the measured θ (in degrees)

$$[\theta] = \theta * 100 * Mr / c * l \quad (3)$$

$$[\theta]_{MRW} = \theta * 100 * Mr / c * l * N_A \quad (4)$$

where θ is the measured ellipticity in degrees, c is the protein concentration in mg/ml, l is the pathlength in cm, and Mr and MRW are the protein molecular weight and mean residue weight,respectively. N_A is the number of amino acids per protein. $[\theta]_{MRW}$ has units degrees * cm^2 * $dmol^{-1}$.The values obtained were normalized by subtracting the baseline recorded for the buffer having the same concentration of denaturant or salt under similar conditions.

2.5.1. Thermal denaturation analysis

The thermal denturation experiments with the individual proteins and the complexes were performed in the above said spectropolarimeter. The temperature was varied using a PFD-425S Peltier temperature controller in the instrument. The ellipticity values were measured at 222 nm. The samples were heated at a constant rate of 1 °C/min in a 2 mm cell and scanned in the temperature range 25–100 °C. The folded fraction of protein at any temperature was determined as follows as also reported earlier in other systems [30]:

$$\left([\theta]^{obs} - [\theta]^{den}\right) / \left([\theta]^{nat} - [\theta]^{den}\right) \quad (5)$$

where, $[\theta]^{obs}$ is the ellipticity at a given temperature, $[\theta]^{den}$ at highest temperature and $[\theta]^{nat}$ at lowest temperature, respectively.

2.5.2. Urea and guanidium hydrochloride denaturation analysis

Chemical denaturation analysis of the PfHsp70-1 was performed by observing the changes in the ellipticity values at 222 nm wavelength. The spectra were calculated in the above defined buffer with the required quantity of the denaturant concentration added and the samples were incubated for 6–8 h. The folded fraction of the protein at each denaturant concentration was calculated using Eq. (5).

2.6. Fluorescence spectroscopy

Fluorescence spectra were recorded with a Perkin-Elmer LS5B spectrofluorometer in a quartz cell with 5 mm path length. 0.5 μM protein was used for all the measurements in a buffer containing 20 mM Tris–HCl pH8.0 and 30 mM NaCl buffer. The denaturants viz., urea (0–8 M) and guanidinium hydrochloride(0–6 M) were added in the same buffer and samples incubated for 6–8 h before recordings. At an excitation walength of 290 nm the samples were scanned for emission from 300–400 nm.

3. Results

3.1. Bioinformatics analysis and domain organization

PfHsp70-1 shares up to 70% homology with its counterparts from other sources (Supplementary Fig. 1A). The Fold Index server (http://bioportal.weizmann.ac.il/fldbin/findex) shows three predominantly folded regions in the protein constituting about 60% of the sequence (Fig. 1A). The individual domains in the protein were identified using JPRED2 [31] and 3DPSSM [32]. The results along with the homology studies led to the demarcation of PfHsp70-1 into three structural regions (Fig. 1B). The N-terminal (1–374) domain which is homologous to the human Hsp70 ATPase domain (PDB ID: 2E88) and residues 1–349 of the bovine Hsc70 (PDB ID: 2QW9) is called the

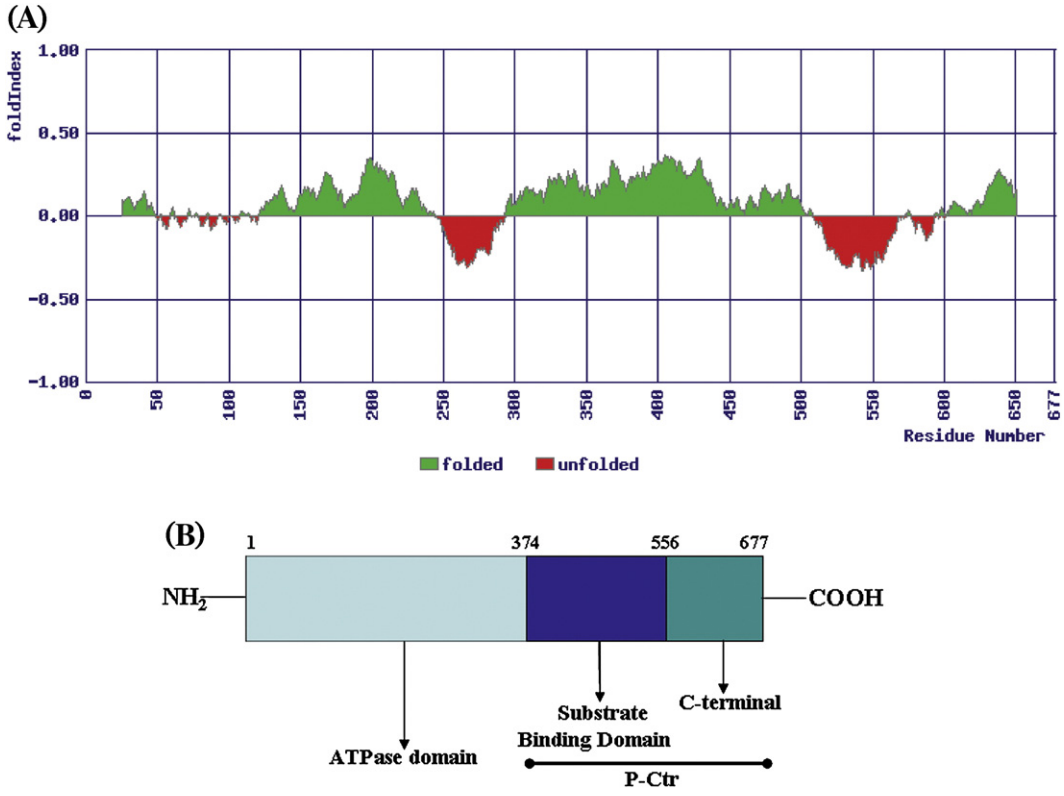

Fig. 1. (A) Fold prediction of PfHsp70-1. Unstructured predicted regions are indicated in *red* while structured regions are shown in *green* (B) Domain organization of PfHsp70-1. P-Ctr refers to the substrate-binding domain and the extended C-terminal region found in *P. falciparum*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

nucleotide-binding domain (NBD), C-terminal end to NBD (374–556) is a substrate or peptide binding domain (SBD) and is similar to the domains found in murine Hsc70 (PDB ID: 1CKR) and *E. coli* DnaK (PDB ID: 1DKY) (Supplementary Fig. 1B, C). A functionally uncharacterized sub-domain (556–677) is also present in the protein, which although has been reported in other sources [33], is comparatively longer in PfHsp70-1.

3.2. Over-expression and purification of PfHsp70-1 and its truncated variants

The full-length *Hsp70-1* gene was PCR amplified, cloned into pET-21d with a C-terminal hexa-histidine tag. It was over expressed in *E. coli* C41 (DE3) containing the RIG plasmid [25] (coding for Arg, Ile, Gly) to overcome rare codon usage. This expression system promotes the translation of the full-length construct as the main product. Purified PfHsp70-1 runs at 75 kDa on 10% SDS-PAGE gels (Supplementary Fig. 2A). The truncated variants NBD (cloned in pET21d), SBD and P-Ctr were cloned into pQE-60 vector with a C-terminal hexa-histidine tag and purified from *E. coli* C41 (DE3) and XLI-Blue cells respectively. The respective domains were purified as described earlier were monitored using SDS-PAGE gels. The domains run as purified bands of approximately 42 kDa, 23 kDa and 35 kDa for NBD, SBD and P-Ctr respectively (Supplementary Fig. 2B, C and D). The molecular weights are in agreement with those expected from the amino-acid sequences. In the size-exclusion chromatography analyses using a Superdex-200 column, the elution volumes of full-length Hsp70-1, SBD (Supplementary Fig. 3A, B) and P-Ctr point towards the existence of higher oligomeric forms while the chromatogram of NBD suggests that it exists as a monomer in solution (Supplementary Fig. 3C). The oligomerization of full-length protein was found to be nucleotide dependent with the loss in the quaternary structure in the presence of ATP (Fig. 2).

3.3. Chaperone assays

PfHsp70-1 is an ATP-dependent chaperone whose molar excess over the aggregated substrate supplemented with an ATP-regeneration system can be efficient for refolding. We have used a five fold molar excess of PfHsp70-1 (protomer to protomer ratio) for refolding of heat-denatured G6PDH and alpha glucosidase. The enzymes were not completely denatured as we were trying to look for the folding efficiency of PfHsp70-1 alone without the presence of other folding partners. Chemically denatured substrates may introduce artifacts in chaperone activity [34] and we therefore used the heat-denatured substrates that are stringent in terms that they do not refold spontaneously as has been shown in previous studies [35,36]. The prolonged heat incubation for 20 min abolished the spontaneous refolding of the substrate as was confirmed by the control without the presence of chaperones. The refolding activity was expressed as percentage gain of activity compared to the activity of denatured enzyme. In the presence of molar excess of PfHsp70-1 alone the refolding experiments resulted in an approximate increase of 15% activity. When the reaction mixture was supplemented with Hsp40 (Pfj1) in the physiological ratio of 5:1 a further 5% increase in the activity was observed, resulting in an overall gain of 20% enzyme activity (Fig. 3). We checked the activity of Pfj1 alone to probe whether the enhancement in the chaperone activity of PfHsp70-1 is due to additive or co-operative effects of Pfj1. The lack of intrinsic chaperone activity in the case of Pfj1 alone suggests that the enhancement of the activity of Pf-Hsp70-1 is likely due to co-operative effects. In order to probe a more direct functional interaction between the proteins we examined whether the ATPase activity of PfHsp70-1 is enhanced in the presence of Pfj1, we found a marginal increase in the ATPase activity of PfHsp70-1 (Supplementary Fig. 4). However, additional experiments involving natural substrates of the protein are necessary for more detailed conclusions on the nature of the interactions. The ratio of the

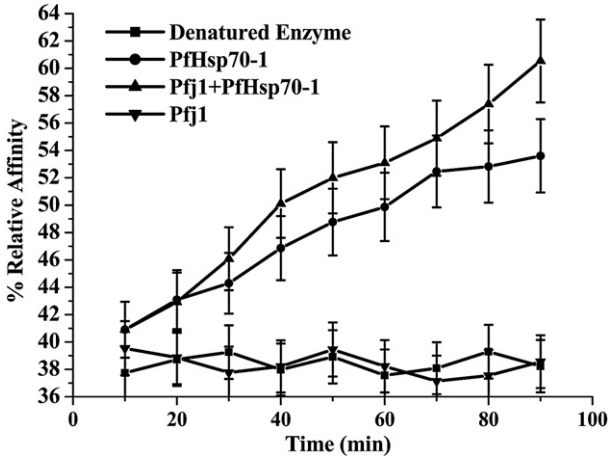

Fig. 3. *In vitro* chaperone assay of PfHsp70-1.No activity is present in denatured enzymes in the absence of chaperone. Presence of PfHsp70-1 in five fold molar excess of denatured substrates (α-glucosidase and G6PDH) regains ~52% activity, which is further enhanced in the presence of Pfj1 (PFD0465w) that does not show any intrinsic chaperone activity.

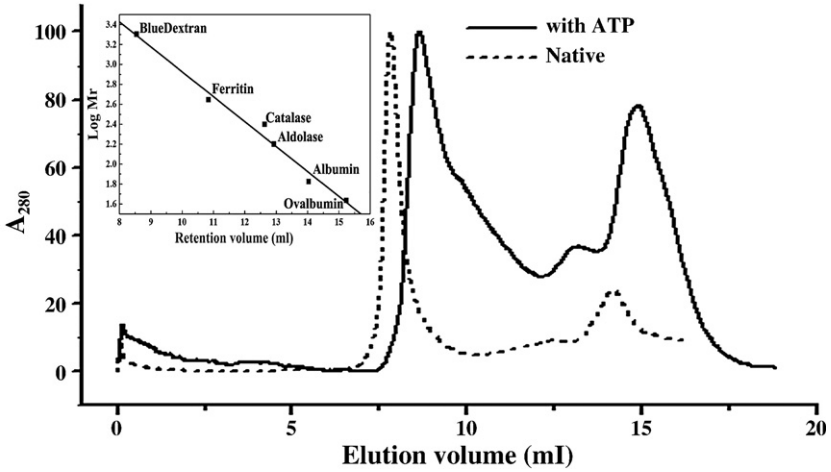

Fig. 2. Size exclusion chromatographic profile of PfHsp70-1. Presence of 3 mM ATP increases the fraction of monomeric species (second peak). *Inset* shows a calibration curve of the column.

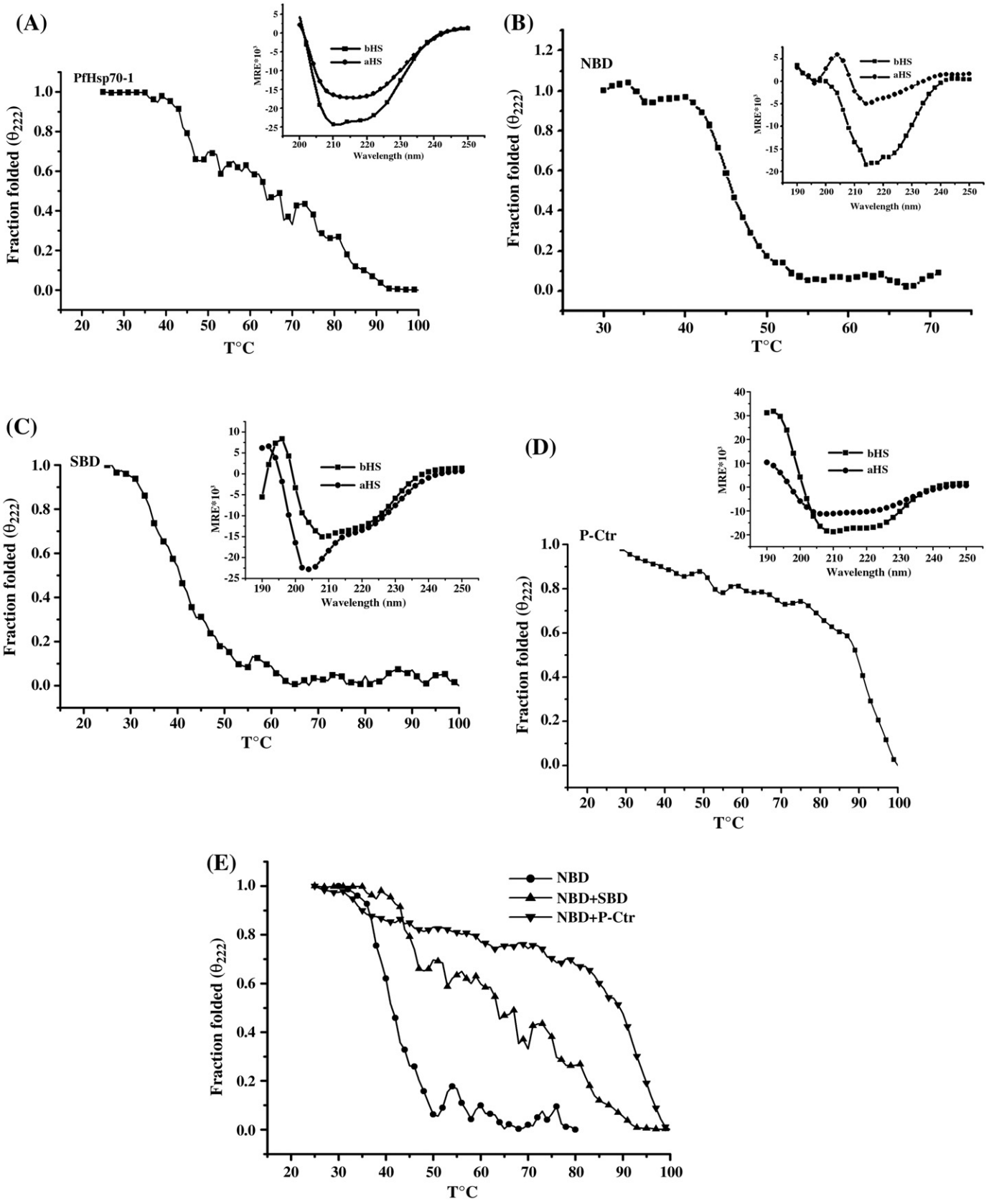

Fig. 4. Thermal stability analysis of native and truncated mutants. (A), (B), (C) and (D) Thermal denaturation profile of PfHsp70-1, NBD, SBD and P-Ctr. *Inset* shows far-UV spectra for each of the proteins before (bHS) and after (aHS) heat denaturation. (E) Folded fraction of NBD, SBD + NBD and P-Ctr + NBD in equimolar ratio as measured by changes in CD ellipticity at 222 nm. Data were fit into Eq. (5).

proteins used was similar to the earlier reports involving Hsp70 and DnaJ (Hsp40 homolog) in *E. coli* [37].

3.4. ATPase activity of full-length PfHsp70-1 and NBD

Most of the functions performed by Hsp70 family of proteins are ATP dependent. The ATPase activity of PfHsp70-1 has been reported earlier [13]. The values of 594.5 μM and 8.9 nmol/min/mg of protein for K_m and V_{max} respectively obtained here for the full-length protein are in agreement with the earlier reports (Supplementary Fig. 4). However, the nucleotide-binding domain alone didn't exhibit ATPase activity in spectrophotometric assays that are less sensitive compared to radio labeled assays. We then utilized changes in intrinsic tryptophan fluorescence to identify the nucleotide-binding properties of the NBD (Supplementary Fig. 5A, B). Subsequently, we used the radioisotope based nucleotide hydrolysis assays where the NBD was found to hydrolyze [γ-^{32}P]ATP in both concentration and time dependent experiments (Supplementary Fig. 5C and D). The ATPase activity was utilized as one of the parameters in our studies for studying the stability of the protein.

3.5. Secondary structure and intrinsic tryptophan fluorescence of PfHsp70-1 and its variants

The secondary structure contents of a protein can be estimated from CD spectroscopy. α-helical proteins are characterized by two minima at 222 nm and 208 nm whereas a predominantly β-sheet protein gives a minima at 216 nm [38]. The secondary structure content of PfHsp70-1 and its variants were estimated from their CD spectra (Supplementary Fig. 6A) using the K2D2 server. All the proteins were found to be predominantly helical in nature with helical content greater than 50%. PfHsp70-1 contains three tryptophan residues at positions 32, 101 and 593. In the truncated variants the first two were located in the ATPase domain and the third one in P-Ctr. The SBD mutant does not contain tryptophan residues. Interestingly all the proteins give an emission maximum centered between 335 and 345 nm (Supplementary Fig. 6B). The buried tryptophan residues in protein give emission maxima around 330–340 nm whereas solvent exposed tryptophan molecules give a maxima around 353 nm [39]. From the emission maxima of full-length and truncated variants it can be concluded that the tryptophan molecules are in buried pockets in a relatively less hydrophilic environment and the proteins are properly folded.

3.6. The extended C-terminal segment contributes importantly to thermal stability of PfHsp70-1

A temperature optimum of 50 °C was observed for ATPase activity of PfHsp70-1 when probed at temperatures ranging from 30–80 °C. Even at higher temperatures of about 80 °C, up to about 20% of the maximal activity was found to be present (Supplementary Fig. 7). In case of NBD a complete loss of activity was observed at temperatures above 30 °C (*data not shown*). Subsequently, we went on to analyze the thermal stability properties of the full-length protein. The truncated versions were used to probe their roles in the protein stability. The changes in the CD ellipticity at 222 nm were monitored from 30–100 °C. The full-length protein exhibits a steep transition from 30–45 °C followed by a gradual loss of $\theta_{222\ nm}$ intensity and another steep transition at 80 °C (Fig. 4A). In the case of NBD, a single sigmoidal transition was observed in the range of 40–50 °C with the midpoint centered around 45 °C suggesting a co-operative unfolding (Fig. 4B). NBD thus can be suggested to undergo a two state unfolding process without the involvement of any folding intermediates. SBD exhibits a transition at about 40 °C, but still retains about 80% of the total structure (Fig. 4C). P-Ctr maintains most of its structure till about 80 °C although a gradual decrease is observed. This is followed by a sharp decrease in the $\theta_{222\ nm}$ intensity (Fig. 4D). The results on the individual domains coupled to the full-length results suggest that in PfHsp70-1, the initial transition and loss in structure at about 40 °C can be attributed to the NBD. The second steep transition at ~80 °C can be attributed to the loss in the structure of the SBD/P-Ctr. P-Ctr as explained before has an additional C-terminal extension compared to SBD. We therefore probed for the stabilizing effects of SBD and P-Ctr on the protein by examining the interactions with the NBD. Gel filtration experiments showed that NBD and SBD/P-Ctr interact with each other in solution (data not shown). At equimolar concentrations, both SBD and P-Ctr were found to increase the folded fraction of NBD in the CD experiments (Fig. 4E). However, the stabilizing effect of the P-Ctr and by extension the extended C-terminal tail is more than double that of the SBD alone. Addition of P-Ctr leads to the maintenance of almost 80% of the secondary structure even at 85 °C, while less than 10% structure is maintained in the case of the SBD added to the NBD. Overall the experiments suggest that the C-terminal segment of the protein contributes significantly towards the stability of the protein, perhaps by interacting closely with the N-terminal domain.

3.7. Effects of urea-induced denaturation probed through activity assays, CD and fluorescence

We followed up the thermal denaturation experiments with chemical denaturation and in the first instance used urea as the denaturant. The ATPase activity of PfHsp70-1 was monitored in the

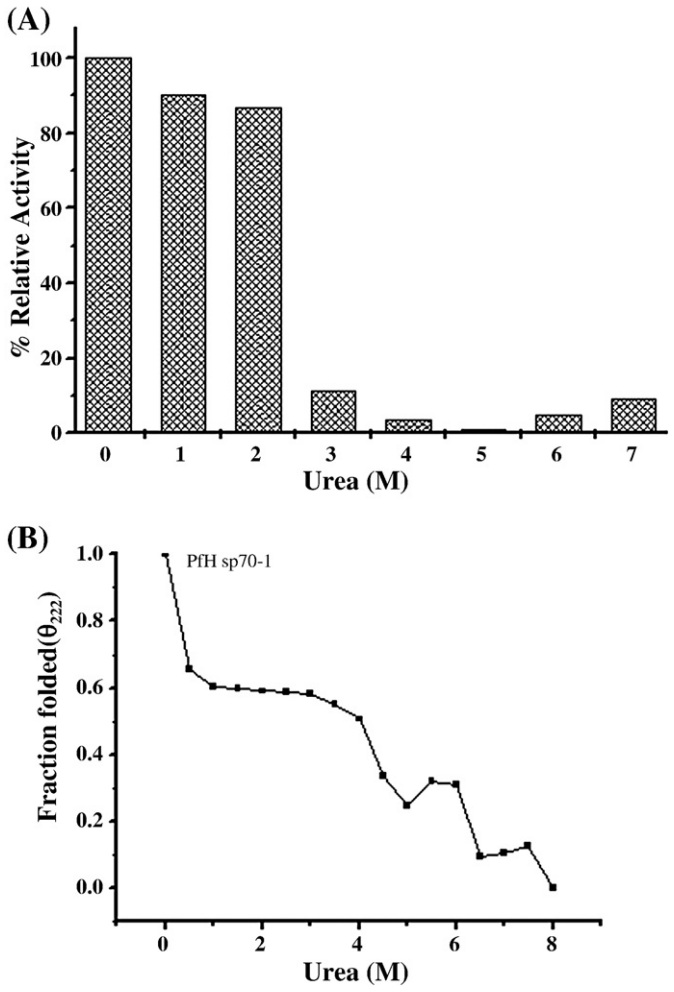

Fig. 5. Effect of urea on native protein activity and structural changes in full-length and truncated variants. (A) Decrease in the ATPase activity of PfHsp70-1 in the presence of different molar concentration of urea. (B) Changes in CD ellipticity at 222 nm for PfHsp70-1 with increase in concentration of urea. Data were fit into Eq. (5). Values in the absence of urea were taken as maximum.

presence of increasing concentrations of urea. The protein exhibits up to 80% activity even at 2 M urea concentration. Subsequently, the activity rapidly diminishes at higher urea concentrations (Fig. 5A).

The structural changes in the protein induced by the addition of the denaturant were followed by examining the intrinsic tryptophan fluorescence and CD experiments. The samples were incubated for 2, 4, 8 and 16 h at 16 °C respectively while the concentration of the denaturant was varied between 0.5 and 4 M. An incubation time of about 8 h was found to be sufficient for attaining equilibrium in the experiments. The urea-induced denaturation of PfHsp70-1 gives a multi-phasic curve (Fig. 5B) analogous to the thermal denaturation experiments. A steep transition was observed till 2 M urea concentration, followed by a gradual loss in the secondary structure till 6 M concentration and a steep transition thereafter.

The changes in the tertiary structure of PfHsp70-1 and its truncated variants were monitored by observing the changes in the intrinsic tryptophan fluorescence in the presence of increasing urea concentrations (Fig. 6). In case of the full-length protein and P-Ctr, a gradual red shift of emission wavelength was observed with both proteins reaching a maximum shift of 350 nm and 351 nm respectively at 7 M urea concentrations. While the transitions in full-length protein are more prominent, P-Ctr appears to undergo fewer changes at the lower urea concentrations. In case of tryptophan a complete solvent exposure of the tryptophan is seen at about 3 M urea concentration which in the presence of equimolar SBD concentration shifts to about 5 M urea concentration. Also in the presence of SBD the transitional changes in wavelength at lower urea concentrations become more incremental compared to those in its absence.

3.8. PfHsp70-1 is more sensitive to guanidium hydrochloride induced denaturation

As with the urea experiments the effects of adding GdmCl were probed through activity assays, CD and fluorescence experiments. The ATPase activity of PfHsp70-1 was monitored in the presence of increasing concentrations of guanidium hydrochloride. About 80% loss of ATPase activity was observed in the presence of only 0.5 M GdmCl concentration (Fig. 7A).

A range of GdmCl concentrations were tested over varying time periods in the CD experiments. A time period of 4 h at 16 °C was found to be adequate for attaining equilibrium and no further changes were observed either in ellipticity or fluorescence spectra after this time period. No phasic transitions are seen in the denaturation curve with GdmCl; instead a gradual loss in secondary structure is observed ending with a complete loss of secondary structure at 6 M concentration (Fig. 7B).

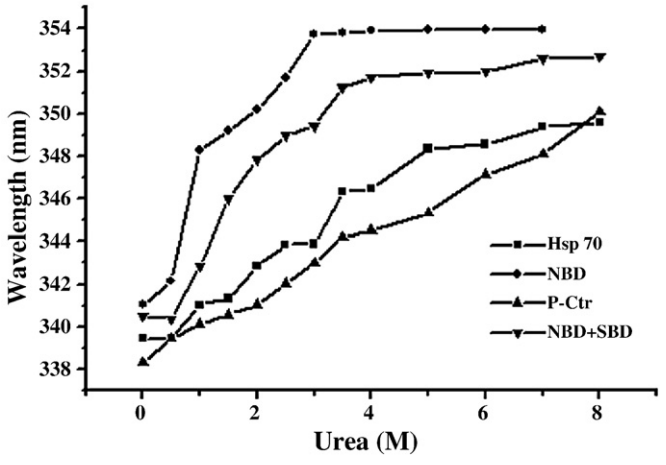

Fig. 6. Red shift in the tryptophan fluorescence emission wavelength of PfHsp70-1 and various truncated mutants on incubation with increasing concentration of urea.

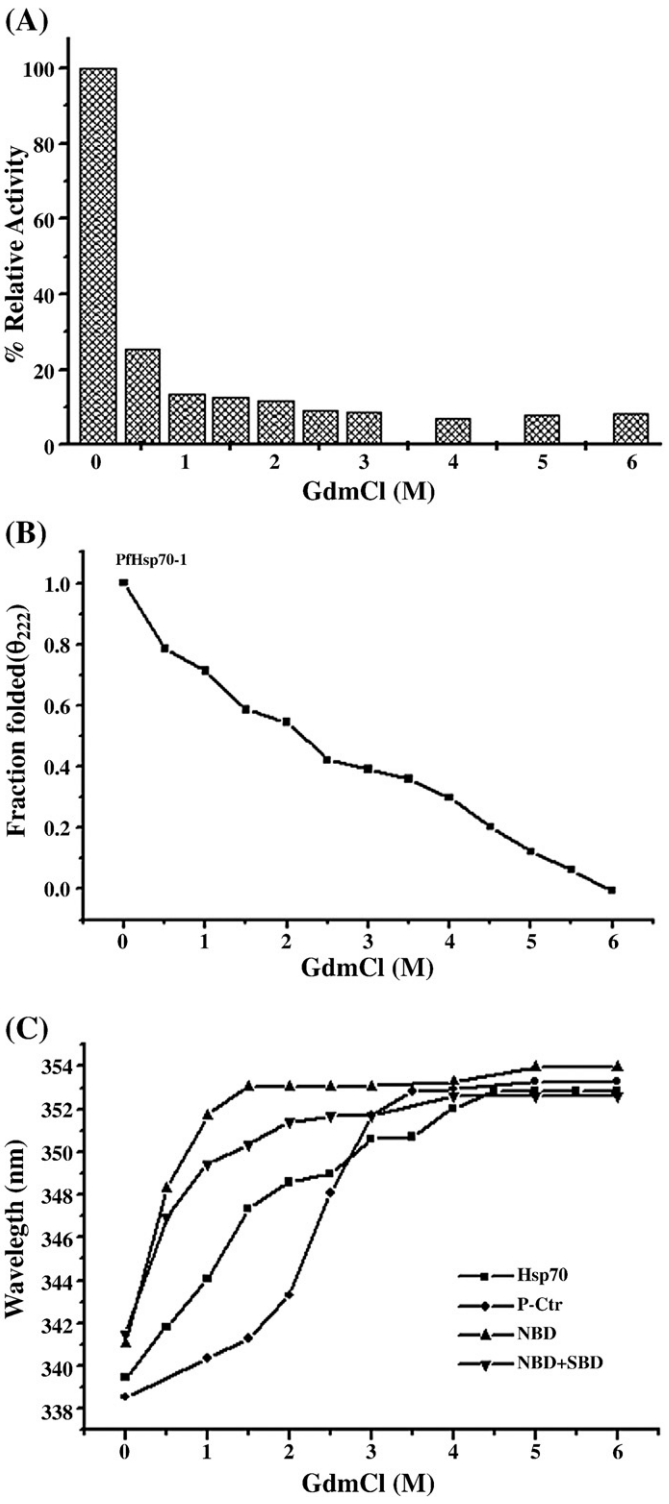

Fig. 7. GdmCl induced changes in full-length protein activity and structure changes in various constructs. (A) Decrease in the ATPase activity of PfHsp70-1 in the presence of increasing concentration of GdmCl. (B) Changes in CD ellipticity at 222 nm for PfHsp70-1 with increase in the concentration of GdmCl. Data were fit into Eq. (5). Values in the absence of GdmCl were taken as maximum. (C) Red shift in the tryptophan fluorescence emission wavelength.

Changes to the intrinsic tryptophan fluorescence of PfHsp70-1 and its truncated variants were monitored to detect structural changes as a result of GdmCl induced denaturation (Fig. 7C). In case of PfHsp70-1, a gradual red shift is observed which reaches a maximum at 352 nm and 4.5 M GdmCl concentration. P-Ctr is relatively more stable in the

presence of GdmCl. In the case of NBD, a fully exposed tryptophan species is observed at 1 M GdmCl concentration and points towards the complete denaturation of this domain. Addition of the SBD makes the NBD more stable as observed by the increased GdmCl concentration (2.5 M) necessary for the denaturation.

Overall the protein is more sensitive to GdmCl induced denaturation compared to urea. However, here also the addition of the C-terminal segment protects the N-terminal segment from early denaturation.

4. Discussion

The *P. falciparum* Hsp70-1 is a multi-domain protein composed of a nucleotide-binding domain, a substrate-binding domain and a C-terminal sub-domain attached to the latter. The truncated mutants exist as oligomers in solution except for the N-terminal nucleotide-binding domain. This suggests that the substrate-binding domain is involved in oligomerization. The oligomerization is partly reversed by nucleotide binding as reported in other members [33].

PfHsp70-1 has drawn a lot of attention recently mainly because of its important role in the translocation of about 500 nuclear encoded parasite proteins to the apicoplast, a relict plastid. Other groups demonstrated subsequently that it also possesses a basal chaperone activity in the absence of ATP that is enhanced in the presence of the nucleotide. In the present work we have confirmed the earlier results and have extended it to provide the first evidence for functional interactions between the PfHsp70-1 and Hsp40 (Pfj1, PFD0465w), which functions as a co-chaperone enhancing its chaperone activity. This is analogous to earlier reports in the homologous proteins DnaJ and DnaK in other bacterial systems like *E. coli* [17]. However, since the parasite suggestedly codes for ~43 Hsp40 homologs, more experimentation involving at least some of them is necessary to map other relevant interactions of PfHsp70-1.

The NBD alone exhibits a relatively weaker ATPase activity pointing towards the role of other domains of the protein in modulating the activity. The temperature optimum of 50 °C for the activity agrees well with earlier suggestions for PfHsp70-1 to be active at higher temperatures encountered during the lifecycle of the parasite. The thermal denaturation curves observed from CD spectroscopy for PfHsp70-1 results from the sum of temperature dependent changes in the unstructured parts and the functional domains of the protein. Among the truncated variants, NBD is characterized by a T_m of 45 °C and is a relatively unstable domain. The SBD doesn't show a marked change in the secondary structure content. It can be concluded that most of the perturbations in the protein structure are local disturbances in the unstructured regions rather than complete denaturation of the protein. The P-Ctr construct contains the extended C-terminal segment. This C-terminal sub-domain of PfHsp70-1 is elongated compared to other known heat shock proteins. Some studies have suggested that it contains functionally important motifs necessary for protein–protein interactions [14]. We propose an additional role for this stretch, in the light of the present work, in enhancing the overall thermostability of the protein as the presence of P-Ctr provides stability to NBD which otherwise is unstable in isolation. PfHsp70-1 shares about 43% sequence homology with *Thermus thermophilus* Hsp70 which has a melting temperature of 110 °C [40]. The extended C-terminus of about 60 residues is a shared characteristic of the PfHsp70-1 and some protozoan parasitic Hsp70 homologs. The results described here could as well apply to this latter class of Hsp70 homologs given the high sequence homology between them.

The presence of considerable secondary structure accounts for the significant ATPase activity of the protein in 2 M urea that subsequently decreases due to a complete loss of secondary structure. A transition observed at 2 M urea can be attributed to the unfolding of NBD and contrasts with the C-terminal portion which is relatively more stable. The stability of the C-terminal portion of the protein is further confirmed from the fluorescence experiments. Whereas the tryptophan of NBD becomes completely solvent exposed at 3 M urea concentration, the full-length protein and P-Ctr tryptophan residues become fully exposed at 7 M urea concentration. The change in the red-shift pattern of NBD in the presence of SBD also points towards the stability SBD. The urea denaturation studies overall reveal that PfHsp70-1 harbors a structurally stable C-terminal sub-domain that contributes significantly to the protein's stability.

In the case of GdmCl, a loss of about 80% ATPase activity is observed even at 0.5 M denaturant. The 20% loss in protein structure is comparable to that observed at 2 M urea concentration. The GdmCl results also broadly mimic those seen with urea denaturation except that the protein is more sensitive to GdmCl. GdmCl is a known inhibitor of predominantly ionic interactions compared to the hydrophobic interactions disrupted by urea. It has also been reported to act as an inhibitor of ATPase activity in the related Hsp104 and at saturating concentrations inhibits up to 50% of the activity [41]. It is certainly possible that GdmCl acts as an inhibitor in the present case also as suggested by the reduced ATPase activity at a concentration less than 2 M where significant unfolding is not observed. The inhibition of about 80% ATPase activity in our case could be a resultant of a combination of inhibition and protein denaturation. Consequently, the chemical denaturation studies suggest that the molecular forces involved within the protein molecule are predominantly ionic in nature as observed from the greater sensitivity towards GdmCl denaturation.

5. Conclusions

We have explored the biophysical properties of PfHsp70-1, an important drug target in malaria research. The C-terminus that was previously known to possess an adjuvant effect, enhancing the DNA vaccine potency has been biophysically characterized for the first time. The studies demonstrate its significant contribution in stabilizing the otherwise unstable nucleotide-binding domain (NBD) of PfHsp70-1. The result is important in the light of the fact that parasite has to face drastic temperature fluctuations during its lifecycle involving two hosts. PfHsp70-1 possesses a higher oligomeric quaternary association that is disrupted only in the presence of ATP. It was found to interact with Pfj1 and represents the first functional characterization of the interactions between these parasite proteins. Overall, the results further our understanding of PfHsp70-1 and expectedly will help exploit it as a novel therapeutic target.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bpc.2009.03.006.

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