Published in final edited form as:

Protein Expr Purif. 2015 February ; 0: 57–65. doi:10.1016/j.pep.2014.09.013.

Overproduction and Biophysical Characterization of Human HSP70 Proteins[†]

Rebba C. Boswell-Casteel, Jennifer M. Johnson, Kelli D. Duggan, Yuko Tsutsui¹, and Franklin A. Hays^{1,2}

¹Department of Biochemistry and Molecular Biology, 940 Stanton L. Young Blvd, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104

²Stephenson Oklahoma Cancer Center, University of Oklahoma Health Sciences Center, Oklahoma City, OK, 73104

Abstract

Heat shock proteins (HSP) perform vital cellular functions and modulate cell response pathways to physical and chemical stressors. A key feature of HSP function is the ability to interact with a broad array of protein binding partners as a means to potentiate downstream response pathways or facilitate protein folding. These binding interactions are driven by ATP-dependent conformational rearrangements in HSP proteins. The HSP70 family is evolutionarily conserved and is associated with diabetes and cancer progression and the etiopathogenesis of hepatic, cardiovascular, and neurological disorders in humans. However, functional characterization of human HSP70s has been stymied by difficulties in obtaining large quantities of purified protein. Studies of purified human HSP70 proteins are essential for downstream investigations of protein-protein interactions and in the rational design of novel family-specific therapeutics. Within this work, we present optimized protocols for the heterologous overexpression and purification of either the nucleotide binding domain (NBD) or the nucleotide and substrate binding domains of human HSPA9, HSPA8, and HSPA5 in either *E. coli* or *S. cerevisiae*. We also include initial biophysical characterization of HSPA9 and HSPA8. This work provides the basis for future biochemical studies of human HSP70 protein function and structure.

[†]Research reported in this study was supported by an Institutional Development Award (IDeA) from the National Institute of General Medical Sciences of the National Institutes of Health under grant number P20GM104934, Experimental Therapeutics Seed Grant from the Stephenson Cancer Center at the University of Oklahoma Health Sciences Center (to F.A.H.), and American Heart Association predoctoral fellowship 13PRE17040024 (to R.C.B.-C.).

© 2014 Elsevier Inc. All rights reserved.

To whom correspondence should be addressed: Franklin A. Hays, ¹Department of Biochemistry and Molecular Biology, 940 Stanton L. Young Blvd, University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA, 73104; Tel.: (405) 271-2227 Ext. 61213; Fax: (405) 271-3092; Franklin-Hays@ouhsc.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

HSP70; Heat shock protein; Enzyme purification; Recombinant protein expression; molecular chaperone

INTRODUCTION

Heat shock proteins (HSPs) play key roles in maintaining cellular homeostasis and driving cell fate in response to physical or chemical stressors. Functional roles assigned to HSP proteins include facilitating nascent protein folding, protein translocation between cellular compartments, regulating assembly and disassembly of protein complexes, and degradation of unstable and/or misfolded proteins ⁽¹⁾. This functional diversity demonstrates a key conserved feature of the broader HSP family – the ability to interact with a wide array of protein ("client") molecules. Regulation of client molecule binding affinity is achieved through conformational changes resulting from ATP binding to distinct sites in HSP proteins. Thus, understanding how HSPs function at the molecular level is instrumental to understanding their broader biological role in modulating cell survival. Functional characterization of human HSPs has been hampered by difficulties associated with large-scale heterologous overproduction of purified protein as a foundation for driving detailed protein biochemical analysis. Indeed, HSP protein expression is closely regulated in cell systems and highly responsive to cell stress ⁽²⁾.

HSPs are classified based on their molecular weight (in kiloDaltons). There are 7 major HSP families: HSP110, HSP100, HSP90, HSP70, HSP60, HSP40, and small HSPs (typically 20 – 25 kDa). The HSP70 family is one of the most evolutionarily conserved protein families ^(3, 4) and found in a wide array of different species ^(5–7). In addition, all eukaryotes have more than one HSP70 encoding gene ⁽⁸⁾. The human HSP70 family consists of at least 13 homologous proteins ⁽⁹⁾ including HSPA5 (Grp78, Bip, or Hsp70-5), HSPA8 (Hsc70, Hsp70-8, or Hsp73), and HSPA9 (mortalin, GRP75, mtHSP75, mtHSP70 or Hsp70-9) ⁽⁸⁾. Conservation of primary sequence extends to functional overlap within the HSP70 family with cellular localization serving, in part, as a means to regulate HSP70 functional activity and role in cell function.

HSPA5 is located predominantly in the endoplasmic reticulum (ER), and serves as a master regulator of the unfolded protein response pathway, folding of ER or secretory proteins, and assembly of protein complexes ^(10, 11). It also has roles in regulating calcium homeostasis ⁽¹²⁾, serves as an ER stress sensor ⁽¹³⁾, and targets misfolded/malfolded proteins for ER associated degradation ⁽¹⁾. There is an accumulation of evidence that HSPA5 has important functions in diabetes ⁽¹⁴⁾, neurological disorders ⁽¹⁵⁾, cancer progression ^(16, 17), and drug resistance ^(18, 19). HSPA8 is constitutively expressed in most tissue types and localizes to the nucleus and cytosol. HSPA8 was discovered as an uncoating ATPase catalyzing the ATP-dependent uncoating of clathrin-coated pits ^(20, 21). Since then, it also has been shown to have roles in maintaining protein homeostasis ⁽²²⁾, regulating protein translocation ^(23, 24), targeting proteins for degradation ^(25, 26), and glucose stimulated insulin secretion ⁽²⁷⁾. HSPA8 is an essential housekeeping gene ⁽²⁸⁾ involved in clinical

diseases including, cancer, cardiovascular, neurological, and hepatic disorders⁽²⁹⁾. A third HSP70 family member, HSPA9, was originally discovered for its contribution to cellular mortality in mouse embryonic fibroblasts (MEFs), by analyzing the cytosolic fraction of serially passaged MEFs^(30, 31). Since then, it has been shown that HSPA9 changes its subcellular localization from the mitochondria, in normal cells, to the cytosol in neoplastic cells⁽³²⁾. HSPA9 has essential roles in the translocation of pre-proteins into the mitochondrial matrix, intracellular trafficking, and receptor internalization⁽³³⁾. As with HSPA5 and HSPA8, HSPA9 function is linked to various cancers, neurological disorders, and diabetes⁽³³⁾.

HSP70 proteins have a conserved domain structure (Figure 1A) consisting of: 1) a nucleotide binding domain that hydrolyzes ATP (NBD), 2) a protease sensitive linker region, 3) a substrate binding domain (SBD), and 4) a G/P-rich C-terminal domain containing an EEVD-motif (excluding HSPA5 and HSPA9), which enables these proteins to bind to other HSPs and co-chaperones^(8, 34, 35). The NBD consists of 2 large domains (I and II), which can be further divided into 2 subdomains (A and B). Domains I and II form a cleft, with IA and IIA lining the base and IB and IIB forming the walls of the nucleotide binding pocket (Figure 1A)^(36–40). The NBD is followed by a short linker domain that couples nucleotide hydrolysis in the NBD to the opening and closing of the substrate binding pocket of the SBD^(41–43). The SBD contains a β -sandwich subdomain (SBD- β) with a hydrophobic groove for client binding and an α -helical region (SBD- α) that forms a substrate binding site “lid” (Figure 1A)^(39, 44). The binding and discharge of client molecules in the SBD is driven by ATP-dependent conformational changes with the ADP-bound state having higher affinity for client molecules^(43, 45). The vast majority of details pertaining to HSP70 function at the molecular level have been derived from studies of prokaryotic homologs such as DnaK^(34, 43, 46). To date, the atomic structure of full-length human HSP70 proteins has remained elusive and a detailed molecular understanding of how ATP binding/hydrolysis is coupled to client binding is not clearly defined. Furthermore, human HSP70 proteins have a high degree of sequence conservation yet display broad functional roles in a cell. Detailed protein biochemical studies in defined *in vitro* systems are required to further characterize human HSP70 function at the molecular level. This characterization includes an immense array of protein-protein interactions (PPIs) mediated by HSP70 proteins and their role in human pathologies. Characterization of PPIs can lead to the development of novel allosteric inhibitors with higher specificity for individual HSP70 proteins⁽²⁹⁾. In the current work, we present protocols for the overexpression and purification of human NBDs or NBD-SBD constructs from human HSPA5, HSPA8, and HSPA9. We also provide initial biophysical characterization of human HSPA8 and HSPA9.

MATERIALS AND METHODS

Molecular Cloning and Plasmid Generation

cDNAs from human HSPA9 (Clone ID: HsCD00080058), HSPA8 (Clone ID: HsCD00040011), and HSPA5 (Clone ID: HsCD000041118) were obtained from the DNASU Plasmid Repository (www.dnasu.org) in a pDONR221 shuttle plasmid. The genes were subcloned from pDONR221 plasmid using the following primers to introduce C(5')-

NcoI and C(3')-BamHI restriction sites into the PCR amplified construct: HSPA9 (residues 47 – 597) - CACCATGGAAGCATCAGAAGCA (forward) and CGGATCCTTCCATCTTGGTTTC (reverse); HSPA8 (residues 4 – 543) ACAACCATGGGAGGACCTGCAGTTGG (forward) and CTCGGGATCCCTCAAGTGAATTCTTG (reverse); HSPA8-NBD (residues 4 – 381) GCCGCCATGGGAGGACCTGCAGTTGGTAG T (forward) and GGCGGATCCAGACAAGATGGCTGCCT (reverse); and HSPA5-NBD (residues 26 – 407) CTCAACCATGGTGGGCACGGTG (forward) ATCATGGATCCACCAGAGAGCAC (reverse). HSPA5 (residues 26 – 563) was cloned via ligation independent cloning, as previously described ⁽⁴⁷⁾, using the following primers: CAAGGACCGAGCAGCCCCCTCAGTGGGCAC GGTGGTCGGC (forward) and ACCACGGGGAACCAACCCTCCGCTTTCCA ACTCATTTCT (reverse). All PCR amplification utilized Phusion DNA Polymerase with PCR products cleaned by isopropanol precipitation. All amplified constructs, except HSPA5, were inserted into a modified pET52b(+) (Novagen) plasmid containing a C-terminal 3C-protease-10x histidine tag.

HSPA5 was inserted into a modified yeast 2μ plasmid containing a C-terminal GSS-3C-protease-8x histidine tag. All plasmid maintenance and propagation was conducted in XL2Blue Ultracompetent *E. coli* cells (Stratagene #200150).

HSPA9, HSPA8, HSPA8-NBD, and HSPA5-NBD Expression

Plasmids were transformed into Rosetta TM(DE3)pLysS (EMD Millipore #70956) competent cells for protein expression. Positive transformants were selected by plating transformed cells on LB plates containing 100 μg / ml ampicillin. 500 ml LB overnight precultures containing 200 μg / ml ampicillin were grown at 37 °C and 200 rpm. Protein expression was carried out by inoculating 2.8 L baffled Fernbach flasks containing 920 ml of ZYM-5052 auto-induction media ⁽⁴⁸⁾ with 80 ml of overnight culture. Cultures were incubated at 37 °C for 4 hours then shifted to 18 °C (HSPA8, HSPA8-NBD, and HSPA5-NBD) or 22 °C (HSPA9) and continued for an additional 14 hours shaking at 200 rpm. Cells were harvested by centrifugation (2500 × g, 30 minutes, 4 °C) and resuspended in 2 ml of Buffer 1 (50 mM HEPES, 20% (v/v) glycerol, 500 mM NaCl, 2 mM PMSF, 10 mM β-mercaptoethanol, pH 8.0 RT) per 1 g of cell pellet (typical yields for each construct:

HSPA9 – 16 g cells / L culture ; HSPA8 – 18 g cells / L culture ; HSPA8-NBD 16 g cells / L culture ; and HSPA5-NBD 11 g cells / L culture). Resuspended cells were used immediately or stored at –20 °C.

HSPA5 Expression

HSPA5 protein expression utilized W303- pep4 (*leu2–3,112 trp1-1 can1–100 ura3-1 ade2-1 his3–11,15 pep4 MATα*) *S. cerevisiae* cells transformed with sheared salmon sperm DNA (Invitrogen #15632-011) . Positive yeast transformants were selected in 1 × synthetic complete histidine dropout media ("SC-HIS") to select for episomal HIS3 expression. 1 × SC-HIS growth media contains 1 × CSM-HIS (Sunrise Science #1023#), 0.67% (w/v) yeast nitrogen base without amino acids (RPI #Y20040), 1% (w/v) D-glucose (Sigma-Aldrich #G8270), and 1% (w/v) D-raffinose (Carbosynth #OR06197) for a final carbohydrate concentration of 2% (w/v). Cells were grown in 10 L working volumes using a Sartorius BIOSTAT Cplus fermentor. Once cells reached an optical density of ~20 (at 600 nm) they

were induced in batch by addition of 1.3 L of 4 × yeast extract-peptone-galactose (YPG) containing 8% (w/v) yeast extract (RPI #Y20020), 16% (w/v) peptone (RPI #P20250), and 8% (w/v) galactose (Sigma-Aldrich #G0625). Cultures were grown continuously at 30 °C and 200 rpm and harvested by centrifuging in 1 L volumes at 3600 × g for 30 min and 4 °C. Cells were resuspended in 3 ml of Buffer 2 (20 mM Tris pH 7.4 RT, 20% (v/v) glycerol, 500 mM NaCl, 2 mM PMSF, 2 mM DHALT protease inhibitor, and 5 mM β-mercaptoethanol) per 1 g of wet cell pellet (HSPA5 cultures averaged 17 g cells / L of culture). Resuspended cells were used immediately or stored at −20°C.

HSPA9, HSPA8, HSPA8-NBD, and HSPA5-NBD Cell Lysis

To resuspended cells add 1 mg/ml lysozyme, 100 units / L DNase, and 3 mM MgCl₂ and nutate for 30 min at 4 °C. Cells were lysed via sonication (Sonics Vibra Cell VCX500, ½” (13 mm) probe 630-0220) at 72% amplitude (30 sec on, 1 min off; 7 cycles) on ice. Cell lysate was spun at 19,000 × g for 90 min at 4 °C. Resuspended HSPA5 expressing yeast cells were lysed using 3 passes at ~28,000 psi in an Avestin C-3 Emulsiflex and centrifuged at 7500 × g for 1 hour at 4 °C.

HSPA8, HSPA8-NBD, and HSPA5-NBD Purification

Supernatant was pooled post lysis and 2 ml of Ni-NTA slurry (50% v/v) was added per 400 ml of cleared lysate and allowed to batch bind at 4 °C for approximately 18 hours. The histidine-tagged protein was then purified via sequential washes with 20 mM HEPES, 500 mM NaCl, 10% (v/v) glycerol, 1 mM β-mercaptoethanol, pH 7.5 RT with either 10 mM imidazole (wash 1, Buffer 3a, 80 ml), 25 mM imidazole (wash 2, Buffer 3b, 50 ml), or 500 mM imidazole (elution, Buffer 3c, 20 ml). Protein was diluted to less than 0.5 OD₂₈₀ / ml and dialyzed for 4 hours at 4 °C into Buffer 4a (20 mM HEPES, 50 mM NaCl, 10% (v/v) glycerol, 1 mM EDTA, 4 mM β-mercaptoethanol, pH 7.4 RT; HSPA5-NBD is sensitive to low salt concentrations). The protein was then loaded onto a 5 ml HiTrap Blue HP column (GE Healthcare #17-0413-01) running at 0.5 ml / min with Buffer 4a and the protein was eluted using a step gradient of 2%, 30%, 50% and 100% Buffer 4b (20 mM HEPES, 2.5 M NaCl, 10% (v/v) glycerol, 1 mM EDTA, 4 mM β-mercaptoethanol, pH 7.4 RT). Peak fractions in the 30% step were pooled, 400 μg of His-MBP-3C protease was added to the pooled protein and dialyzed into 300 mM NaCl, 10% (v/v) glycerol, 4 mM β-mercaptoethanol, and either 20 mM HEPES, pH 7.4 RT (Buffer 5, HSPA8-NBD, and HSPA5-NBD) or 20 mM CAPS, pH 10.0 RT (Buffer 6, HSPA8) for approximately 10 hours. Following dialysis, the C-terminal expression tag and His-MBP-3C protease were removed with the addition of 1 mL of TALON cobalt resin (50% v/v slurry, Pierce #89965) to the protein and nutated at 4 °C for 1 hour (as the 3C protease itself has a histidine tag that is thrombin, not 3C, cleavable). The protein/resin mixture was then poured into a gravity column and the flow-through was collected. The protein was concentrated using a 30,000 MWCO (molecular weight cutoff) centrifugal concentrator (Millipore #UFC903096).

HSPA9 Refolding and Purification

The resultant pellet following cell lysis was resuspended in 5 ml of Buffer 7 (40 mM Tris-HCl, 500 mM NaCl, 3.5 M guanidine HCl, 1 mM PMSF, pH 8.5 RT) per gram of cell pellet

for denaturation. The resuspended pellet was then sonicated (Sonics Vibra Cell VCX500, ½” (13mm) probe 630-0220) at 60% amplitude (30 sec on, 1 min off; 7 cycles). The mixture was spun at $27,000 \times g$ for 40 minutes at 4 °C. A gravity column was packed with 30 ml of TALON cobalt resin (50% v/v slurry, Pierce #89965) and equilibrated with 50 ml of Buffer 7. Supernatant was passed over the packed column 4–6 times then washed with 40 mM Tris-HCl, 500 mM NaCl, 3.5 M guanidine HCl, pH 8.5 RT 20 mM imidazole (Buffer 8a, 120 ml, wash) or 400 mM imidazole (Buffer 8b, 30 ml, elution). Supernatant was repassed, washed, and eluted an additional 5 to 6 times to remove the remaining HSPA9 from the flow through. Eluted protein (2 – 15 mg denatured protein / ml buffer) was then dialyzed into Buffer 9 (55 mM Tris-HCl, 2.0 M guanidine HCl, pH 8.2 RT) for approximately 10 hours. Protein was removed from dialysis and diluted to 1 – 3 mg / ml with fresh Buffer 9. Buffer 10 (55 mM Tris-HCl, 500 mM L-arginine, 21 mM NaCl, 0.88 mM KCl) was utilized for protein refolding and aliquoted at 100 mL per 10 ml of protein solution (1 – 3 mg / ml) and placed into a separatory funnel. The protein solution was placed into a beaker with gentle stirring at room temperature. Buffer 10 was titrated into the protein solution using a slow steady dropwise addition over several hours with gentle stirring (light precipitation will be observed, if precipitation is heavy slow the addition of the Buffer 10) and the refolding was allowed to continue for 10 – 12 hours at room temperature. Protein was then filtered with a 0.45 µm filter to remove precipitation. Protein was concentrated using a stirred cell concentrator (30,000 MWCO, Millipore #PLTK04310) until total volume reached approximately 30 – 50 ml and then dialyzed for 10 – 12 hours in Buffer 11 (55 mM Tris-HCl, 250 mM NaCl, 10% (v/v) glycerol, 1 mM EDTA, 1 mM KCl, 4 mM β-mercaptoethanol, pH 8.4 RT). A subsequent dialysis step was then performed to exchange the protein into Buffer 12a (55 mM Tris-HCl, 50 mM NaCl, 10% (v/v) glycerol, 1 mM EDTA, 1 mM KCl, 4 mM β-mercaptoethanol, pH 8.4 RT) for 2 hours at 4 °C immediately before use.

The protein was then loaded onto a 5 ml HiTrap Blue HP column (GE Healthcare #17-0413-01) running at 0.5 ml / min with Buffer 12a and the protein was eluted using a step gradient of 2%, 10%, 50% and 100% Buffer 12b (55 mM Tris-HCl, 2.5 M NaCl, 10% (v/v) glycerol, 1 mM EDTA, 1 mM KCl, 4 mM β-mercaptoethanol, pH 8.4 RT). Peak fractions in the 50% step were pooled, 400 µg of His-MBP-3C protease was added to the pooled protein and dialyzed into Buffer 13 (20 mM CAPS, 300 mM NaCl, 10% (v/v) glycerol, 1 mM KCl, 4 mM β-mercaptoethanol, pH 10.0 RT). The C-terminal expression tag and His-MBP-3C protease were removed as described above. Purified and refolded

HSPA9 was concentrated by placing it in a dialysis bag on a bed of solid PEG 20,000 with gentle rocking. HSPA9 has been successfully concentrated up to 10.5 mg / ml. (176.6 µM in 500 µl) via PEG 20,000. We purpose HSPA9 can be concentrated further using PEG 20,000 because it has been concentrated as high as 25 mg / ml (420 µM in 600 µl) using a 30,000 MWCO centrifugal concentrator. However, a centrifugal concentrator is not recommended because HSPA9 binds to the regenerated cellulose membrane of the concentrator (Table 1).

HSPA5 purification

Supernatant was recovered from centrifugation and incubated with Ni-NTA resin (50% v/v) overnight in batch at 4°C with gentle rocking. The histidine-tagged protein was purified from total protein via sequential 10 column volume washes in 20 mM HEPES pH 7.5, 500 mM NaCl, 10% (v/v) glycerol, 1 mM β -mercaptoethanol, and 2 mM DHALT protease inhibitor (Buffer 14) containing 10 mM imidazole (wash 1, Buffer 14a) or 25 mM imidazole (wash 2, Buffer 14b) and eluted from the resin with 5 ml 250 mM imidazole (elution, Buffer 14c). Eluted protein was dialyzed overnight in 20 mM HEPES pH 7.5, 100 mM NaCl, 10% (v/v) glycerol, 1 mM β -mercaptoethanol, and 2 mM DHALT protease inhibitor (Buffer 15). The C-terminal expression tag was removed using a 5-fold excess of target protein with His-MBP-3C protease followed by application to a TALON cobalt resin, as described above. The column flow-through was collected in 10 ml total volume. Cleaved HSPA5 was then further purified using a HiTrap Blue HP column as described above (see "HSPA8, HSPA8-NBD, and HSPA5-NBD Purification") using a linear gradient from 100 mM to 500 mM NaCl with peak fractions pooled and dialyzed overnight into Buffer 6. The purified protein was then concentrated using a 50,000 MWCO (Millipore #UFC905096) centrifugal concentrator to 10 mg / ml and stored in 10% (v/v) glycerol at -80 °C (Table1).

Circular Dichroism (CD) Spectroscopy

CD spectra were obtained using a JASCO J-715 spectropolarimeter equipped with a PTC-348WI temperature controller, and a sealed 0.1 cm cuvette. The scan rate was set at 10 nm / min scanning from 190 – 260 nm, with a 1 nm bandwidth, 25 °C, and 3 repetitions.

HSPA8 utilized a molar concentration of 7.35 μ M, and 12.4 μ M for HSPA8-NBD in 20 mM NaH₂PO₄, 200 mM NaCl, 5% (v/v) glycerol, pH 7.4 RT. Thermal unfolding of

HSPA8 and HSPA8-NBD was carried out with a heating rate of 1 °C / min from 25 °C to 55 °C while monitoring CD signal at 222 nm. HSPA9 was analyzed at 16 μ M protein concentration in 20 mM NaH₂PO₄ pH 7.4, 100 mM NaCl, and 5% (v/v) glycerol. Thermal unfolding of HSPA9 was carried out with a heating rate of 2 °C / min from 25 °C to 80 °C while monitoring CD signal at 222 nm.

ATP Hydrolysis Assay

The ability of HSPA9 to hydrolyze ATP was determined using the EnzCheck® Phosphate Assay Kit (Molecular Probes # E6646) following the manufacturer's instructions. Briefly, in the presence of inorganic phosphate, the substrate MESG (2-amino-6-mercapto-7-methyl-purine) is enzymatically converted to ribose 1-phosphate and 2-amino-6-mercapto-7-methyl-purine by purine nucleoside phosphorylase. This conversion results in a spectrophotometric shift in the wavelength of maximum absorbance from 330 nm to 360 nm. A standard curve was generated using inorganic phosphate concentrations ranging from 2 μ M to 160 μ M in the presence of 5 μ M ATP. Standard reactions utilized a 75 μ M (HSPA9) protein concentration and 5 μ M ATP. All reactions took place at room temperature.

Analytical ultracentrifugation (AUC)

HSPA9, HSPA8, and HSPA8-NBD oligomerization state and conformation were evaluated at 0.3 and 0.9 OD₂₈₀ / ml protein loading concentration using analytical

ultracentrifugation sedimentation velocity. The experiments were performed in a Beckman Optima XL-I analytical ultracentrifuge at the Center for Analytical Ultracentrifugation of Macromolecular Assemblies at the University of Texas Health Science Center at San Antonio. The sample was measured at 40,000 rpm and scanned at 280 nm in intensity mode in 20 mM CAPS pH 10, 200 mM NaCl, 1 mM MgCl₂, and 1 mM TCEP (HSPA9) or 20 mM HEPES pH 7.4, 300 mM NaCl, 5% (v/v) glycerol, 4 mM β-mercaptoethanol, and 1 mM MgCl₂ (HSPA8 and HSPA8-NBD). Experimental data were collected at 20 °C using 1.2 cm epon 2-channel centerpieces (Beckman-Coulter). Hydrodynamic corrections for buffer density and viscosity were estimated by UltraScan ⁽⁴⁹⁾ to be 1.002930 g / ml and 1.01295 cP. AUC Data Analysis: All data were analyzed with UltraScan-III ver. 2.2, release 1743 as previously described ⁽⁵⁰⁾. Diffusion-corrected integral sedimentation coefficient distributions were obtained from the enhanced van Holde – Weischet analysis ⁽⁵¹⁾.

Dynamic Light Scattering (DLS)

DLS intensities were measured for HSPA9 (50 mM CAPS, 200 mM NaCl, 1 mM KCl, 5% (v/v) glycerol, 4 mM β-mercaptoethanol, pH 10), HSPA8 (Buffer 6), and HSPA8-NBD (Buffer 5) using a Zetasizer Nano Z instrument (Malvern Instruments, UK). Data acquisition and manipulation was performed using Zetasizer Nano software. All data were collected using automated settings at 25 °C (HSPA8 and HSPA8-NBD) or 23 °C (HSPA9) a Zen2112 cuvette, a 173° scattering angle, and reported values are the mean of 3 replicates.

RESULTS AND DISCUSSION

Protein Overexpression and Purification

HSPA9, HSPA8, HSPA8-NBD, and HSPA5-NBD were cloned into a modified pET52b(+) plasmid for recombinant expression in Rosetta TM(DE3)pLysS *E. coli* cells, while HSPA5 was expressed as a C-terminal 8-histidine fusion construct in W303- pep4 cells using a yeast episomal galactose inducible system as outlined above. As previously stated by Luo, *et al.*, ⁽⁵²⁾ HSPA9 is heavily expressed in inclusion bodies and sediments into the insoluble fraction following cell lysis. Therefore, aggregated HSPA9 must be solubilized, extracted, and refolded into its native conformation for downstream studies. Like Luo, *et al.*, ⁽⁵²⁾ we used the chaotropic agent guanidine HCl for solubilization and denaturation of HSPA9. While, Luo, *et al.*, ⁽⁵²⁾ used 2 M concentrations of guanidine HCl we found that increasing the concentration to 3.5 M increased the efficiency of IMAC purification and reduced the amount of irreversibly bound protein to the resin (data not shown). We also substituted Co-NTA resin for Ni-NTA resin as we observed a 40% higher binding capacity in a head-to-head comparison with Ni-NTA (data not shown). We tried the refolding protocol outlined by Luo, *et al.*, ⁽⁵²⁾, but were unsuccessful in obtaining mg quantities of HSPA9 due to extremely heavy protein precipitation. Altering the addition rate of the refolding buffer, stirring speed during refolding, refolding temperature, or decreasing / increasing the initial concentration of HSPA9 resulted in no significant increases in yield. Our observed discrepancies with the published purification protocol may stem from location of the fusion partner, N- vs. C-terminus, or from the expression of different residues (52 – 679 vs. 47 – 597). Therefore, we developed an additional refolding protocol to facilitate HSPA9 purification as described above and outlined in Figure 2. Most

notably, our refolding buffer (Buffer 10) contains 500 mM L-arginine, and by utilizing a dilution refolding method our final concentration of guanidine HCl is 0.2 M. L-arginine and low concentrations of guanidine HCl have been shown to stabilize proteins during refolding and prevent protein aggregation^(53–57). We also included an additional affinity purification step utilizing Cibacron Blue resin.

HSPA8, HSPA8-NBD, HSPA5, and HSPA5-NBD were isolated from the soluble fraction following cell lysis and initially purified using Ni-NTA resin. The purification of all 4 constructs (except HSPA5) converged at the second affinity purification step - Cibacron Blue (Figure 2). Cibacron Blue is a biomimetic dye that has been used for the affinity purification of proteins containing an intact dinucleotide fold^(58–60) and was recently reported for the purification of the NBD of HSPA9⁽³⁹⁾. Since Cibacron Blue is specific for intact dinucleotide folds⁽⁵⁹⁾ we utilized this purification step to isolate protein targets with intact NBD domains. Cibacron Blue resin has also been shown to isolate apoenzymes containing the dinucleotide fold⁽⁶¹⁾. We hypothesized that purification with the Cibacron Blue resin would allow us to purify properly folded, catalytically active proteins with vacant NBD binding sites. The purification of active proteins with empty NBD binding sites is important for future studies of small molecules that exert their effects by binding to the NBD active site of these proteins. We were able to purify all 5 of our constructs to > 95% purity (Figure 3) and with the final yields outlined in Table 2. Absence of nucleotide binding in the ATP binding pocket of the NBD of HSPA8-NBD was verified by x-ray crystallography (data not shown).

Circular Dichroism and Thermal Denaturation

To further characterize the recombinant HSPA9, HSPA8, and HSPA8-NBD circular dichroism and thermal stability tests were performed. The far UV CD spectra at 25 °C showed two minima at 221 nm and 206 nm, and a maximum at 195 nm for HSPA9 (Figure 4A). Thermal stability tests showed that HSPA9 maintained stability up to 50 °C, had a transition temperature range of 55 – 85 °C, and a T_m of 71.8 °C (Figure 4B). The broad thermal transition observed for HSPA9 is indicative of a negatively cooperative denaturation process for the protein and supports a conformationally disperse population being present prior to protein unfolding. This is further supported by the AUC results discussed below (Figure 5). Given the gradual melting of HSPA9, and lack of observable precipitation after heating the sample to 110 °C, we hypothesized that HSPA9 had high thermostability and would spontaneously refold upon cooling. Therefore, the sample was allowed to return to 25 °C and another CD spectrum was taken. The post-heated sample showed two minima at 220 nm and 207 nm, with a maximum at 200 nm, indicating retention of fold and thermal denaturation of HSPA9 was a reversible process. HSPA8 and HSPA8-NBD had similar spectra with two minima at 219 nm, 211 nm (HSPA8, Figure 4C) and 220 nm, 212 nm (HSPA8-NBD, Figure 4E), and maximum at 200 nm (HSPA8, Figure 4C) and 204 nm (HSPA8-NBD, Figure 4E). Similarly, HSPA8 and HSPA8-NBD was stable up to 40 °C, transitioned between 43 – 49 °C, and had a T_m of 45.5 °C (HSPA8, Figure 4D) and 45.7 °C (HSPA8-NBD, Figure 4F).

Analytical Ultracentrifugation and Dynamic Light Scattering

The van Holde – Weischet integral distribution plot showed a homogenous narrow distribution for HSPA8 and HSPA8-NBD with an average *s*-value of 3.1S for HSPA8-NBD and 3.5S for HSPA8 (Figure 5A) demonstrating a monomeric and monodisperse distribution of protein in solution. The calculated frictional coefficient (f/f_0) for HSPA8-NBD is 1.42 and for HSPA8 is 1.52 indicating both proteins are more globular in shape with a measured weight average molar mass for each sample of 40.9 kDa (95% CI: 23.2, 57.9) for HSPA8-NBD (41.6 kDa theoretical molar mass) and 64.7 kDa (95% CI: 38.5, 90.9) for HSPA8 (59.4 kDa theoretical molar mass). Confidence intervals were determined from iterative Monte Carlo simulations and reflect approximate noise in the empirical data. In contrast, van Holde – Weischet analysis of HSPA9 sedimentation velocity data produced a wide distribution of determined *s*-values ranging from 2.67 – 5.82S (Figure 5A). DLS experiments produced average hydrodynamic diameters of 21.3 nm (HSPA9), 12.9 nm (HSPA8), and 11.2 nm (HSPA8-NBD) (Figure 5b), with a corresponding polydispersity of 26% (HSPA9), 30% (HSPA8), and 31% (HSPA8-NBD). The larger R_H observed for HSPA9 is consistent with the increased frictional coefficient obtained from AUC and supports an argument that HSPA9 adopts an extended conformation or conformational distribution in solution. Full-length human HSPA9 has been refractory to structure determination efforts, including in our own hands, and this conformational variability may play a key role in preventing the formation of well ordered crystals for high-angle x-ray data collection.

HSPA9 ATPase Activity Assay

In order to verify the ability of Cibacron Blue purification to isolate active protein, an ATPase activity assay was performed on HSPA9. We utilized the same protein:ATP ratio as previously reported⁽⁵²⁾. However, we did not quench the reactions with a trichloroacetic acid solution as described by Luo, *et al.*, because we found that trichloroacetic acid interfered with UV-Vis measurements obtained at 360 nm in a concentration dependent manner (data not shown). HSPA9 was capable of hydrolyzing ATP (Figure 6). Kinetic parameters for single site HSPA9-mediated ATP hydrolysis were obtained by fitting the resultant curve of the formation of inorganic phosphate over time to a first-order exponential function. HSPA9 yielded an observed first-order rate constant for the hydrolysis of ATP, k_{obs} , of $36.5 \times 10^{-4} \pm 1.4 \times 10^{-4} \text{ s}^{-1}$, which is approximately 6-fold higher than the reported value of $6.0 \times 10^{-4} \pm 0.7 \times 10^{-4} \text{ s}^{-1}$ by Luo, *et al.*⁽⁵²⁾.

CONCLUSIONS

This work presents a novel, detailed, protocol for the overproduction and purification of 3 human HSP70 proteins: HSPA9, HSPA8, and HSPA5. Initial biophysical characterization of HSPA9 and HSPA8 produced by the described methods is also presented and demonstrates that catalytically active HSPA9 adopts an extended conformation, with increased conformational polydispersity, relative to HSPA8, in solution. Human HSP70 proteins have been implicated in a wide range of disease states including cancer, diabetes, cardiovascular, neurological, and hepatic disorders. Dissecting these disease states and the development and tuning of novel therapeutics will be significantly advanced through studies centered on

purified HSP70 proteins (e.g., regulation of the unfolded protein response pathway by HSPA5 in the ER). In summary, this work provides a basis for future pharmaceutical development, kinetic characterization, and biophysical analysis of the human heat shock proteins HSPA9, HSPA8, and HSPA5.

Acknowledgments

We thank Dr. Doris Benbrook at the University of Oklahoma Health Sciences Center for helpful comments and suggestions during the course of these studies. We acknowledge Dr. Borries Demeler and Virgil Schirf at the University of Texas Health Science Center for assistance with AUC data collection and analysis and the Center for Analytical Ultracentrifugation of Macromolecular Assemblies at the University of Texas Health Science Center for performing AUC experiments.

Abbreviations used

HSP	Heat Shock Protein
ER	endoplasmic reticulum
MEF	mouse embryonic fibroblast
NBD	nucleotide binding domain
SBD	substrate binding domain
MWCO	molecular weight cutoff
CD	circular dichroism
AUC	analytical ultracentrifugation
DLS	dynamic light scattering
SC-HIS	synthetic complete histidine dropout media
YPG	yeast extract-peptone-galactose

REFERENCES

1. Athanasiou D, Kosmaoglou M, Kanuga N, Novoselov SS, Paton AW, Paton JC, Chapple JP, Cheetham ME. BiP prevents rod opsin aggregation. *Mol. Biol. Cell.* 2012; 23:3522–3531. [PubMed: 22855534]
2. Benjamin IJ, McMillan DR. Stress (heat shock) proteins: molecular chaperones in cardiovascular biology and disease. *Circ. Res.* 1998; 83:117–132. [PubMed: 9686751]
3. Gupta RS, Singh B. Phylogenetic analysis of 70 kD heat shock protein sequences suggests a chimeric origin for the eukaryotic cell nucleus. *Curr. Biol.* 1994; 4:1104–1114. [PubMed: 7704574]
4. Lindquist S, Craig EA. The heat-shock proteins. *Annu. Rev. Genet.* 1988; 22:631–677. [PubMed: 2853609]
5. Pelham HR. Hsp70 accelerates the recovery of nucleolar morphology after heat shock. *EMBO J.* 1984; 3:3095–3100. [PubMed: 6441707]
6. Li GC, Li LG, Liu YK, Mak JY, Chen LL, Lee WM. Thermal response of rat fibroblasts stably transfected with the human 70-kDa heat shock protein-encoding gene. *Proc. Natl. Acad. Sci. USA.* 1991; 88:1681–1685. [PubMed: 1705702]
7. Radford NB, Fina M, Benjamin IJ, Moreadith RW, Graves KH, Zhao P, Gavva S, Wiethoff A, Sherry AD, Malloy CR, Williams RS. Cardioprotective effects of 70-kDa heat shock protein in transgenic mice. *Proc. Natl. Acad. Sci. USA.* 1996; 93:2339–2342. [PubMed: 8637874]

8. Daugaard M, Rohde M, Jaattela M. The heat shock protein 70 family: Highly homologous proteins with overlapping and distinct functions. *FEBS Lett.* 2007; 581:3702–3710. [PubMed: 17544402]
9. Kampinga HH, Hageman J, Vos MJ, Kubota H, Tanguay RM, Bruford EA, Cheetham ME, Chen B, Hightower LE. Guidelines for the nomenclature of the human heat shock proteins. *Cell stress & chaperones.* 2009; 14:105–111. [PubMed: 18663603]
10. Gidalevitz T, Stevens F, Argon Y. Orchestration of secretory protein folding by ER chaperones. *Biochim. Biophys. Acta.* 2013; 1833:2410–2424. [PubMed: 23507200]
11. Wang M, Wey S, Zhang Y, Ye R, Lee AS. Role of the unfolded protein response regulator GRP78/BiP in development, cancer, and neurological disorders. *Antioxid. Redox Signal.* 2009; 11:2307–2316. [PubMed: 19309259]
12. Coe H, Michalak M. Calcium binding chaperones of the endoplasmic reticulum. *Gen. Physiol. Biophys.* 28 Spec No Focus. 2009:F96–F103.
13. Hetz C. The unfolded protein response: controlling cell fate decisions under ER stress and beyond. *Nat. Rev. Mol. Cell Biol.* 2012; 13:89–102. [PubMed: 22251901]
14. Harding HP, Ron D. Endoplasmic reticulum stress and the development of diabetes: a review. *Diabetes.* 2002; 3(51 Suppl):S455–S461. [PubMed: 12475790]
15. Zhao L, Longo-Guess C, Harris BS, Lee JW, Ackerman SL. Protein accumulation and neurodegeneration in the woozy mutant mouse is caused by disruption of SIL1, a cochaperone of BiP. *Nat. Genet.* 2005; 37:974–979. [PubMed: 16116427]
16. Dong D, Ni M, Li J, Xiong S, Ye W, Virrey JJ, Mao C, Ye R, Wang M, Pen L, Dubeau L, Groshen S, Hofman FM, Lee AS. Critical role of the stress chaperone GRP78/BiP in tumor proliferation, survival, and tumor angiogenesis in transgene-induced mammary tumor development. *Cancer Res.* 2008; 68:498–505. [PubMed: 18199545]
17. Li J, Lee AS. Stress induction of GRP78/BiP and its role in cancer. *Curr. Mol. Med.* 2006; 6:45–54. [PubMed: 16472112]
18. Ranganathan AC, Zhang L, Adam AP, Aguirre-Ghiso JA. Functional coupling of p38-induced up-regulation of BiP and activation of RNA-dependent protein kinase-like endoplasmic reticulum kinase to drug resistance of dormant carcinoma cells. *Cancer Res.* 2006; 66:1702–1711. [PubMed: 16452230]
19. Reddy RK, Mao C, Baumeister P, Austin RC, Kaufman RJ, Lee AS. Endoplasmic reticulum chaperone protein GRP78 protects cells from apoptosis induced by topoisomerase inhibitors: role of ATP binding site in suppression of caspase-7 activation. *J. Biol. Chem.* 2003; 278:20915–20924. [PubMed: 12665508]
20. Chappell TG, Welch WJ, Schlossman DM, Palter KB, Schlesinger MJ, Rothman JE. Uncoating ATPase is a member of the 70 kilodalton family of stress proteins. *Cell.* 1986; 45:3–13. [PubMed: 2937542]
21. Goldfarb SB, Kashlan OB, Watkins JN, Suaud L, Yan W, Kleyman TR, Rubenstein RC. Differential effects of Hsc70 and Hsp70 on the intracellular trafficking and functional expression of epithelial sodium channels. *Proc. Natl. Acad. Sci. USA.* 2006; 103:5817–5822. [PubMed: 16585520]
22. Ziemienowicz A, Zylicz M, Floth C, Hubscher U. Calf thymus Hsc70 protein protects and reactivates prokaryotic and eukaryotic enzymes. *J. Biol. Chem.* 1995; 270:15479–15484. [PubMed: 7797540]
23. Chirico WJ, Waters MG, Blobel G. 70K heat shock related proteins stimulate protein translocation into microsomes. *Nature.* 1988; 332:805–810. [PubMed: 3282179]
24. Hollenbeck PJ, Saxton WM. The axonal transport of mitochondria. *J. Cell Sci.* 2005; 118:5411–5419. [PubMed: 16306220]
25. Hirasaka K, Tokuoka K, Nakao R, Yamada C, Oarada M, Imagawa T, Ishidoh K, Okumura Y, Kishi K, Nikawa T. Cathepsin C propeptide interacts with intestinal alkaline phosphatase and heat shock cognate protein 70 in human Caco-2 cells. *J. Physiol. Sci.* 2008; 58:105–111. [PubMed: 18307834]
26. Tanaka K, Namba T, Arai Y, Fujimoto M, Adachi H, Sobue G, Takeuchi K, Nakai A, Mizushima T. Genetic evidence for a protective role for heat shock factor 1 and heat shock protein 70 against colitis. *J. Biol. Chem.* 2007; 282:23240–23252. [PubMed: 17556362]

27. Yan FF, Pratt EB, Chen PC, Wang F, Skach WR, David LL, Shyng SL. Role of Hsp90 in biogenesis of the beta-cell ATP-sensitive potassium channel complex. *Mol. Biol. Cell.* 2010; 21:1945–1954. [PubMed: 20427569]
28. Florin L, Becker KA, Sapp C, Lambert C, Sirma H, Muller M, Streeck RE, Sapp M. Nuclear translocation of papillomavirus minor capsid protein L2 requires Hsc70. *J. Virol.* 2004; 78:5546–5553. [PubMed: 15140951]
29. Liu T, Daniels CK, Cao S. Comprehensive review on the HSC70 functions, interactions with related molecules and involvement in clinical diseases and therapeutic potential. *Pharmacol. Ther.* 2012; 136:354–374. [PubMed: 22960394]
30. Wadhwa R, Kaul SC, Ikawa Y, Sugimoto Y. Identification of a novel member of mouse hsp70 family. Its association with cellular mortal phenotype. *J. Biol. Chem.* 1993; 268:6615–6621. [PubMed: 8454632]
31. Wadhwa R, Kaul SC, Mitsui Y, Sugimoto Y. Differential subcellular distribution of mortalin in mortal and immortal mouse and human fibroblasts. *Exp. Cell. Res.* 1993; 207:442–448. [PubMed: 8344392]
32. Wadhwa R, Takano S, Robert M, Yoshida A, Nomura H, Reddel RR, Mitsui Y, Kaul SC. Inactivation of tumor suppressor p53 by mot-2, a hsp70 family member. *J. Biol. Chem.* 1998; 273:29586–29591. [PubMed: 9792667]
33. Kaul SC, Deocaris CC, Wadhwa R. Three faces of mortalin: a housekeeper, guardian and killer. *Exp. Gerontol.* 2007; 42:263–274. [PubMed: 17188442]
34. Mayer MP, Bukau B. Hsp70 chaperones: cellular functions and molecular mechanism. *Cell Mol. Life Sci.* 2005; 62:670–684. [PubMed: 15770419]
35. Chang YW, Sun YJ, Wang C, Hsiao CD. Crystal structures of the 70-kDa heat shock proteins in domain disjoining conformation. *J. Biol. Chem.* 2008; 283:15502–15511. [PubMed: 18400763]
36. Harrison CJ, Hayer-Hartl M, Di Liberto M, Hartl F, Kuriyan J. Crystal structure of the nucleotide exchange factor GrpE bound to the ATPase domain of the molecular chaperone DnaK. *Science.* 1997; 276:431–435. [PubMed: 9103205]
37. Wisniewska M, Karlberg T, Lehtio L, Johansson I, Kotenyova T, Moche M, Schuler H. Crystal structures of the ATPase domains of four human Hsp70 isoforms: HSPA1L/Hsp70-hom, HSPA2/Hsp70-2, HSPA6/Hsp70B', and HSPA5/BiP/GRP78. *PloS one.* 2010; 5:e8625. [PubMed: 20072699]
38. Flaherty KM, DeLuca-Flaherty C, McKay DB. Three-dimensional structure of the ATPase fragment of a 70K heat-shock cognate protein. *Nature.* 1990; 346:623–628. [PubMed: 2143562]
39. Amick J, Schlanger SE, Wachnowsky C, Moseng MA, Emerson CC, Dare M, Luo WI, Ithychanda SS, Nix JC, Cowan JA, Page RC, Misra S. Crystal structure of the nucleotide-binding domain of mortalin, the mitochondrial Hsp70 chaperone. *Protein Sci.* 2014; 23:833–842. [PubMed: 24687350]
40. Macias AT, Williamson DS, Allen N, Borgognoni J, Clay A, Daniels Z, Dokurno P, Drysdale MJ, Francis GL, Graham CJ, Howes R, Matassova N, Murray JB, Parsons R, Shaw T, Surgenor AE, Terry L, Wang Y, Wood M, Massey AJ. Adenosine-derived inhibitors of 78 kDa glucose regulated protein (Grp78) ATPase: insights into isoform selectivity. *J. Med. Chem.* 2011; 54:4034–4041. [PubMed: 21526763]
41. Swain JF, Dinler G, Sivendran R, Montgomery DL, Stotz M, Gierasch LM. Hsp70 chaperone ligands control domain association via an allosteric mechanism mediated by the interdomain linker. *Mol. Cell.* 2007; 26:27–39. [PubMed: 17434124]
42. Jiang J, Prasad K, Lafer EM, Sousa R. Structural basis of interdomain communication in the Hsc70 chaperone. *Mol. Cell.* 2005; 20:513–524. [PubMed: 16307916]
43. Vogel M, Mayer MP, Bukau B. Allosteric regulation of Hsp70 chaperones involves a conserved interdomain linker. *J. Biol. Chem.* 2006; 281:38705–38711. [PubMed: 17052976]
44. Wang H, Kurochkin AV, Pang Y, Hu W, Flynn GC, Zuiderweg ER. NMR solution structure of the 21 kDa chaperone protein DnaK substrate binding domain: a preview of chaperone-protein interaction. *Biochemistry.* 1998; 37:7929–7940. [PubMed: 9609686]
45. Vogel M, Bukau B, Mayer MP. Allosteric regulation of Hsp70 chaperones by a proline switch. *Mol. Cell.* 2006; 21:359–367. [PubMed: 16455491]

46. Jordan R, McMacken R. Modulation of the ATPase activity of the molecular chaperone DnaK by peptides and the DnaJ and GrpE heat shock proteins. *J. Biol. Chem.* 1995; 270:4563–4569. [PubMed: 7876226]
47. Li M, Hays FA, Roe-Zurz Z, Vuong L, Kelly L, Ho CM, Robbins RM, Pieper U, O'Connell JD 3rd, Miercke LJ, Giacomini KM, Sali A, Stroud RM. Selecting optimum eukaryotic integral membrane proteins for structure determination by rapid expression and solubilization screening. *J. Mol. Biol.* 2009; 385:820–830. [PubMed: 19061901]
48. Studier FW. Protein production by auto-induction in high density shaking cultures. *Protein Expr. Purif.* 2005; 41:207–234. [PubMed: 15915565]
49. Demeler B, Gorbet G, Zollars D, Dubbs B, Brookes E, Cao W, Demeler B, Gorbet G, Zollars D, Dubbs B, Brookes E, Cao W. Ultrascan-III version 2.2: A comprehensive data analysis software package for analytical ultracentrifugation experiments. 2014
50. Demeler B. Methods for the design and analysis of sedimentation velocity and sedimentation equilibrium experiments with proteins. Chapter 7, Unit 7 13. *Curr. Protoc. Protein Sci.* 2010
51. Demeler B, van Holde KE. Sedimentation velocity analysis of highly heterogeneous systems. *Anal. Biochem.* 2004; 335:279–288. [PubMed: 15556567]
52. Luo WI, Dizin E, Yoon T, Cowan JA. Kinetic and structural characterization of human mortalin. *Protein Expr. Purif.* 2010; 72:75–81. [PubMed: 20152901]
53. Arakawa T, Tsumoto K. The effects of arginine on refolding of aggregated proteins: not facilitate refolding, but suppress aggregation. *Biochem. Biophys. Res. Commun.* 2003; 304:148–152. [PubMed: 12705899]
54. Singh SM, Panda AK. Solubilization and refolding of bacterial inclusion body proteins. *J. Biosci. Bioeng.* 2005; 99:303–310. [PubMed: 16233795]
55. Yasuda M, Murakami Y, Sowa A, Ogino H, Ishikawa H. Effect of additives on refolding of a denatured protein. *Biotechnol. Prog.* 1998; 14:601–606. [PubMed: 9694682]
56. Tischer A, Lilie H, Rudolph R, Lange C. L-arginine hydrochloride increases the solubility of folded and unfolded recombinant plasminogen activator rPA. *Protein Sci.* 2010; 19:1783–1795. [PubMed: 20665695]
57. Zarrine-Afsar A, Mittermaier A, Kay LE, Davidson AR. Protein stabilization by specific binding of guanidinium to a functional arginine-binding surface on an SH3 domain. *Protein Sci.* 2006; 15:162–170. [PubMed: 16373478]
58. Clonis YD, Labrou NE, Kotsira VP, Mazitsos C, Melissis S, Gogolas G. Biomimetic dyes as affinity chromatography tools in enzyme purification. *J. Chromatogr. A.* 2000; 891:33–44. [PubMed: 10999623]
59. Thompson ST, Cass KH, Stellwagen E. Blue dextran-sepharose: an affinity column for the dinucleotide fold in proteins. *Proc. Natl. Acad. Sci. USA.* 1975; 72:669–672. [PubMed: 164664]
60. Thompson ST, Stellwagen E. Binding of Cibacron blue F3GA to proteins containing the dinucleotide fold. *Proc. Natl. Acad. Sci. USA.* 1976; 73:361–365. [PubMed: 174104]
61. Thompson ST, Cass R, Stellwagen E. A quantitative procedure for recovery of nucleoside phosphate ligands and preparation of apoprotein from holoproteins using blue dextran--Sephadex chromatography. *Anal. Biochem.* 1976; 72:293–296. [PubMed: 182033]

- Protocol for overexpression and purification of HSPA9, HSPA8, and HSPA8
- Biophysical characterization of HSPA9 and HSPA8
- HSPA9 adopts an extended conformation

FIGURE 1. Schematic of HSP70 protein and constructs used

A) Representative cartoon of an HSP70 protein. The NBD is depicted in ovals: domain IA (green), domain IIA (magenta), domain IB (red), and domain IIB (yellow). The NBD is connected to the SBD via a flexible linker (black). The SBD contains a β -sandwich subdomain (SBD- β , orange) and an α -helical region (SBD- α , cyan). B) Schematic of fusion constructs demonstrating location of fusion partner, cleavage site, and amino acid residues expressed.

FIGURE 2. HSP70 expression and purification workflow comparison

The flowchart above provides a graphical description of the expression and purification pipeline for each construct (HSPA9 - blue, HSPA8 - orange, HSPA5 - red, HSPA8-NBD - green, and HSPA5-NBD – magenta). Specific buffers used at each step are indicated by number – refer to the *Materials and Methods* section for specific buffer descriptions.

FIGURE 3. Purification of HSP70 proteins

Cibacron Blue dye affinity column traces with corresponding SDS-PAGE gel of the peak fraction for A) HSPA9, B) HSPA8, C) HSPA5, and D) HSPA8-NBD. Traces were constructed by plotting the 280 nm absorbance values on the ordinate and the increasing salt gradient overtime on the abscissa (the pre-gradient, protein loading, portion of each trace is not shown). The HSPA5-NBD trace was excluded due to high sequence homology and because the purification process mirrored that of HSPA8-NBD.

FIGURE 4. Circular dichroism and thermal unfolding of HSPA9, HSPA8, and HSPA8-NBD. CD spectra of HSPA9 (A, red- pre-heating, blue- post-thermal melting), HSPA8 (C), and HSPA8-NBD (E) were obtained at 25 °C plotting molar ellipticity (Deg·cm² / dmol) on the ordinate and wavelength (nm) on the abscissa. Transition curves of the thermal denaturation of HSPA9 (B), HSPA8 (D), and HSPA8-NBD (F) were acquired by monitoring changes at in molar ellipticity (Deg·cm² / dmol) at 222 nm as a function of increasing temperature (HSPA9 25 °C – 110 °C ; HSPA8 25 °C – 55 °C ; and HSPA8-NBD 25 °C – 55 °C). T_m values were obtained by fitting the data to a four-parameter logistic curve and verified by a

first-derivative plot (data not shown). The vertical line marks the location of the T_m on each transition curve. All fits and graphs were generated using version 6.0e of GraphPad PRISM 6 for Macintosh.

Figure 5. Distribution plots of sedimentation coefficients and hydrodynamic diameters for HSP70 proteins in solution

A) van Holde – Weischet integral distribution plot of HSPA9 (blue), HSPA8 (red), and HSPA8-NBD (green). The corresponding diffusion-corrected integral sedimentation coefficient is noted next to each label. B) DLS measured mean intensities are plotted as a log function of the hydrodynamic diameter (nm). Each protein is colored coded as described in panel A and the average hydrodynamic diameter is noted for each protein. The reported DLS values are the mean of 3 replicates.

FIGURE 6. ATP hydrolysis by HSPA9

Single site kinetic parameters were obtained by plotting the formation of inorganic phosphate as a function of time (0 – 6000 seconds) and fitting the resulted curve to a first-order exponential function to obtain the first-order rate constant. The corresponding rate constant (k_{obs}) and R^2 value is denoted for the fit. Fitting and the resultant graph was generated using GraphPad PRISM version 6.0e.

Table 1

Notable troubleshooting tips and critical steps for specific steps of HSP70 proteins listed under the “Protein” column.

Protein	Purification Step	Critical Steps / Purification Notes
HSPA9	IMAC Purification	Multiple binding and elution steps will be required.
	Protein Refolding	Slow the addition of Buffer 10 if heavy precipitation is observed. Confirm 1–3 mg / ml starting protein concentration.
	Sequential Buffer Exchange	Arginine is slow to dialyze out, and HSPA9 is sensitive to low salt concentrations.
	Dye Affinity Column	The protein will not bind to the column if salt or arginine concentration is too high. The HSPA9 that elutes in the 10% peak has altered ATP hydrolysis and has an optimal pH of 5.5 for protein concentration instead of 10.
	Concentration of Purified Protein	Optimal pH for concentration is 10.0 (Buffer 13). Protein will bind to centrifugal membranes so concentrate in dialysis tubing on a bed of PEG 20,000.
HSPA8 HSPA8-NBD & HSPA5-NBD	Post IMAC Buffer Exchange	Proteins are sensitive to low salt concentration – especially HSPA5-NBD.
	Dye Affinity Column	Proteins will not bind to the column if salt concentration is too high. Protein targets bind best prior to removal of C-terminal His tag.
	Concentration of Purified Protein	Concentrate proteins slowly. HSPA8 is sensitive to lower pH. Optimal pH is 10 (Buffer 6).
HSPA5	All Purification Steps	Protein is unstable at pH < 6.0
	Dye Affinity Column	Protein will not bind to column if salt concentration is too high.

Table 2

Purification Summary

Protein	Purification Step	Recovery (mg / L culture)	Recovery (mg / g wet cells)	Estimated Purity (%)
HSPA9	IMAC	65	4.1	80
	Refolding	45	2.8	90
	Dye Affinity Column *	40	2.5	95
HSPA8	IMAC	4.6	0.26	90
	Dye Affinity Column	1	0.056	98
HSPA8-NBD	IMAC	3.6	0.23	95
	Dye Affinity Column	2	0.13	98
HSPA5	IMAC	4.1	0.31	90
	Dye Affinity Column	2.2	0.11	95
HSPA5-NBD	IMAC	2.5	0.23	75
	Dye Affinity Column	1.5	0.14	95

* Recovery based on protein eluted in the 2nd peak (50% Buffer 12b) only.