RICE UNIVERSITY

The *Saccharomyces cerevisiae* hsp70 escort protein Hep1 exhibits cross-reactivity with a non-cognate chaperone.

by

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ABSTRACT

The *Saccharomyces cerevisiae* hsp70 escort protein Hep1 exhibits cross-reactivity with a non-cognate chaperone.

by

Crystal Stanworth

Genetic studies have provided evidence that the hsp70 escort protein Hep1 is essential for maintaining the solubility of the mitochondrial chaperones Ssc1 and Sq1 in *Saccharomyces cerevisiae*. However, little is known about its metal binding thermostability, and substrate specificity. To characterize these properties, I have demonstrated that the four conserved cysteines in the zinc-finger motif of Hep1 are required for solubility and zinc binding. Mutation of any one of these cysteines to serine abolished soluble expression in *Escherichia coli*. In addition, elemental analysis of purified Hep1 revealed near stoichiometric levels of zinc in purified Hep1. This zinc could be displaced by the sulphydryl-reactive agent methyl methanethiosulfonate (MMTS) implicating the cysteines in the zinc motif as ligands for zinc. A possible interaction between yeast Hep1 and human mitochondrial hsp70 (mthsp70) was also examined by measuring the influence of Hep1 on the solubility of mthsp70 overexpressed in *E. coli*. Human mthsp70 was found predominantly in the soluble fraction upon co-expression with yeast Hep1, whereas mthsp70 was completely insoluble in the absence of Hep1. This interaction is thought to be mediated by the mthsp70 ATPase domain since a mthsp70 truncation mutant having only its N-terminal ATPase domain was insoluble when overexpressed in *E. coli*. To examine whether Hep1 influences mthsp70 thermostability, the effects of temperature on the CD spectra of purified mthsp70 and
Hep1 were investigated. Hep1 exhibited a high melting temperature ($T_m \geq 70^\circ C$), whereas mthsp70 displayed two transitions ($T_m^1 = 48^\circ C; T_m^2 = 78^\circ C$), which were not dramatically altered in the presence of Hep1. Additional studies characterized the solubility and metal content of the small J-type co-chaperone, HscB, which is thought to regulate the ATPase activity of mthsp70. Unlike yeast HscB orthologs, human HscB contains a domain with a tetracysteine motif. Human HscB was shown to be localized to the mitochondria and was found to be soluble when overexpressed in *E. coli*. *Magnetospirillum magneticum* HscB, which contains an N-terminal Cys-rich domain similar to that of human HscB, was shown to coordinate an iron cofactor in an oxygen-sensitive manner. These findings provide evidence that Hep1 requires bound zinc to maintain its solubility, that the hsp70 ATPase domain is responsible for the low solubility of mthsp70, and implicate a role for escort proteins in regulating the functions of metazoan chaperones. Additionally, they implicate a role for the Cys-rich domain of HscB in metal coordination.
ACKNOWLEDGEMENTS

Throughout my time spent at Rice University there have been many people who have given me assistance and advice. I would like to thank my advisor, Dr. Jonathan Silberg, for being my mentor, giving me the opportunity to try my hand at many scientific techniques, and guiding me through the research process. I would also like to thank my committee members, Dr. John Olson, Dr. Richard Gomer, and Dr. Pernilla Wittung-Stafshede for their constructive criticisms and support of my research. Additionally, I would like to express my appreciation for my wonderful lab members and the members of Dr. Matthews' lab, Peter Nguyen, Peng Zhai, Rui Li, Shirley Liu, Dr. Sarah Bondos, Frances Liu, Jia Xu, Jennifer Jamison, George Huang, Taylor Stevenson, and Tina Chen as interaction with them provided not only ideas for solving research problems but a great social environment which made lab work more enjoyable. And last, I would like to thank my husband, Brian Stanworth, for his endless support of my goals and for all the nights he sat in the lab with me while I did this research.
TABLE OF CONTENTS

ABSTRACT...........................................................................................................ii

ACKNOWLEDGEMENTS....................................................................................iv

TABLE OF CONTENTS.......................................................................................v

LIST OF FIGURES AND TABLES......................................................................viii

CHAPTER 1. BACKGROUND AND INTRODUCTION.............................................1

1.1 70-kDa Heat Shock Protein (hsp70).............................................................1

1.2 Eukaryotic mitochondrial hsp70s.................................................................3

1.3 Cochaperone regulation of hsp70 proteins....................................................5

1.4 Human mitochondrial hsp70.........................................................................6

1.5 hsp70 escort protein (Hep1)......................................................................7

1.6 The small J-type cochaperone HscB............................................................11

1.7 Significance..............................................................................................15

CHAPTER 2. MATERIALS AND METHODS.......................................................18

2.1 Hep1 plasmid design, construction, and generation of mutants...............18

2.2 mthsp70 plasmid design, construction, and generation of truncations.....18

2.3 Human HscB plasmid design and construction.......................................20

2.4 Magnetospirillum magneticum HscB plasmid design and construction....20

2.5 Protein expression....................................................................................21

2.6 Expression of Hep1 in M9 minimal media with zinc supplementation......22

2.7 SDS-PAGE analysis of yeast Hep1 and human mHsp70 protein solubility...22

2.8 Purification of S. cerevisiae Hep1-His......................................................22
2.9 Purification of human mthsp70 ........................................ 23
2.10 Purification of human HscB(71-236) ................................ 23
2.11 Purification of *Magnetospirillum magneticum* HscB ........ 24
2.12 Zinc determination ....................................................... 24
2.13 UV-VIS absorbance spectroscopy .................................... 25
2.14 Circular dichroism spectroscopy .................................... 25
2.15 Analytical size exclusion chromatography ....................... 25
2.16 Cell Culture ............................................................. 26

CHAPTER 3. HEP1 AND MTHSP70 RESULTS ................................. 27

3.1 *Saccharomyces cerevisiae* Hep1 cysteines are required for solubility 27
3.2 Zinc content of Hep1 ..................................................... 27
3.3 *S. cerevisiae* Hep1 thermal stability ............................... 32
3.4 *S. cerevisiae* Hep1 promotes human mthsp70 solubility .......... 37
3.5 Purification of recombinant human mthsp70 ....................... 37
3.6 *S. cerevisiae* Hep1 affects human mthsp70 thermal unfolding .... 40
3.7 Domain requirements for mthsp70 insolubility ................... 42

CHAPTER 4. HSCB RESULTS ..................................................... 46

4.1 Human HscB is a mitochondrial protein .......................... 46
4.2 Recombinant human HscB is a soluble monomeric and α-helical protein 46
4.3 *M. magneticum* HscB contains a chromophore .................. 48

CHAPTER 5. DISCUSSION AND PERSPECTIVE ............................ 52

5.1 *S. cerevisiae* Hep1 and human mthsp70 ............................ 52
5.2 Human HscB and *M. magneticum* HscB ............................ 54
5.2 Perspectives...........................................................................................................55

REFERENCES..............................................................................................................58
LIST OF FIGURES

CHAPTER 1

Figure 1.1. hsp70 chaperone architecture.................................................................2
Figure 1.2. Cartoon of the ATPase reaction cycle of hsp70 chaperones.........................4
Figure 1.3. Sequence alignment of Hep1 from eukaryotes.............................................9
Figure 1.4. Core domain of Hep1 NMR solution structure.............................................12
Figure 1.5. Cartoon schematic for chaperone assisted iron-sulfur cluster biogenesis............................13
Figure 1.6. HscB domain structure and multiple sequence alignment of the N-terminus of HscB..........................................................14

CHAPTER 2

CHAPTER 3

Figure 3.1. Hep1 cysteines are required for solubility...............................................28
Figure 3.2. Zinc is required for soluble heterologous expression of Hep1......................30
Figure 3.3. Determination of S. cerevisiae Hep1 purity..............................................31
Figure 3.4. Determination of S. cerevisiae Hep1 metal content by ICP-AES..................33
Figure 3.5. The stoichiometry of Hep1 to zinc is 1:1.....................................................34
Figure 3.6. CD spectrum for Hep1 indicates little secondary structure.........................35
Figure 3.7. Hep1 is extremely thermostable.................................................................36
Figure 3.8. S. cerevisiae Hep1 maintains the solubility of human mthsp70......................38
Figure 3.9. Determination of human mthsp70 purity....................................................39
Figure 3.10. CD spectra for mthsp70...........................................................................41
Figure 3.11. The change in ellipticity for the thermal transitions of mthsp70 and mthsp70 with Hep1 ................................................................. 43
Figure 3.12. Domain requirements for mthsp70 insolubility .................................... 44

CHAPTER 4

Figure 4.1. Human HscB localizes to the mitochondria ..................................... 47
Figure 4.2. Human HscB(71-236) is a highly α-helical and monomeric protein .......... 49
Figure 4.3. mmHscB has a chromophore bound on purification ............................ 51

CHAPTER 5

Figure 5.1. A cartoon schematic for the proposed regulatory role of the Cys-rich domain of mmHscB in iron-sulfur cluster biosynthesis ............................... 56

LIST OF TABLES

Table 2.1 Summary of Expression Constructs .................................................. 19
CHAPTER 1

BACKGROUND AND INTRODUCTION

1.1 70-kDa Heat Shock Protein (hsp70)

The hsp70 chaperones constitute a family of proteins which play a critical role in maintaining cellular function by reversibly binding hydrophobic sequences in their substrate proteins (Mayer, M.P. and B. Bukau, 2005). hsp70s interact with a wide variety of substrate proteins and are responsible for mediating numerous cellular processes including Fe/S cluster biogenesis, protein folding, protein translocation, stress response, and vesicular trafficking (reviewed in Mayer, M.P. and B. Bukau, 2005; Vickery, L.E. and J.R. Cupp-Vickery, 2007).

Figure 1.1 illustrates the basic architecture of hsp70 chaperones. hsp70 molecular chaperones have two domains, a highly conserved N-terminal ATPase domain (~45 kDa) responsible for the catalytic activity of ATP hydrolysis, and a C-terminal peptide-binding domain (PBD; ~25 KDa) that binds diverse peptide substrates. The conformational changes induced by ATP hydrolysis are coupled to changes in the PBD substrate affinity (Laufen, T. et al. 1999). The ATPase domain has an actin-like structure, which is composed of four smaller domains that form a deep catalytic cleft that binds ATP (or ADP and P_i), two potassium ions, and a single magnesium ion. The binding cleft is opened and closed by tilting and sheering motions between these domains (Zhang, Y. and E.R.P. Zuiderweg, 2004). The PBD is composed of two subdomains, the β-sandwich and C-terminal α-helical lid, which function together to bind specific highly conserved and directional hydrophobic patches in their substrate proteins (Cupp-Vickery, J.R. et al. 2004; Rudiger, S., et al. 2001; Zhu, X. et al 1996).
Figure 1.1. **hsp70 chaperone architecture.** *Top*, a domain structure diagram for hsp70 with ATPase domain (red) and PBD (blue). *Bottom*, ribbon diagram of hsp70 illustrating the secondary structure with the ATPase domain (red), PBD (blue), substrate protein (green) with labeled nucleotide binding site, linker, and C-terminal lid. Image was created using Rasmol [PDB 1DKG and 1DKZ] from (Harrison, C.J. et al. 1997 and Zhu, X. et al. 1996, respectively).
Figure 1.2 shows a cartoon of the hsp70 ATPase reaction cycle. ATP-bound hsp70 has a lower affinity and faster exchange rate for substrate proteins compared with other conformational states (Palleros, D.R. et al. 1991). ATP hydrolysis induces a conformational change that locks the substrate into the binding site of the PBD (Palleros, D.R. et al. 1991). As a result, the ADP-bound form has an increased affinity and slower exchange rate which confines the substrate for folding or other function in this state (Palleros, D.R. et al. 1991; Takeda, S. and D.B. McKay, 1996). Subsequent dissociation of ADP and P_i allows ATP to bind again, releasing the substrate and restarting the cycle (Palleros, D.R. et al. 1993). This reaction cycle of the hsp70-type chaperones is also controlled by accessory proteins including J-type cochaperones (Russell, R. et al. 1999) and nucleotide exchange factors, such as GrpE and Bag-1, which serve to stimulate the relatively weak ATP hydrolysis activity of hsp70s (Szabo, A. et al. 1994; Sondermann, H. et al. 2001).

1.2 Eukaryotic mitochondrial hsp70s

hsp70 chaperones are essential for maintaining functional mitochondria as they are responsible for mediating protein translocation through the inner membrane, Fe-S cluster biogenesis, and preventing protein aggregation (reviewed in Lister, R. et al. 2005; Vickery, L.E. and J.R. Cupp-Vickery, 2007; Mayer, M.P. and B. Bukau, 2005). *S. cerevisiae* have two hsp70-type chaperones (Ssc1 and Ssq1) to execute these functions, while humans have a single mitochondrial hsp70 (designated mthsp70, HspA9b, Grp75, and mortalin).

A majority of our understanding of mitochondrial chaperones has come from studies of yeast chaperones. Ssc1 is the predominant hsp70 found in yeast mitochondria.
Figure 1.2. Cartoon of the ATPase reaction cycle of hsp70 chaperones. *Clockwise from top left*, chaperone reaction cycle for the prototypical hsp70 DnaK. First, ATP binding leads to a low affinity state for substrate. Second, DnaJ recruits the substrate protein to the chaperone. Third, ATP hydrolysis converts the complex to the high affinity state for substrate protein. Fourth, GrpE binds to initiate nucleotide exchange. Finally, ATP binds to restart the cycle.
Ssc1 is an essential protein, which participates in the translocation of peptides by the TIM23 complex through association on the matrix side of the membrane with TIM44, an essential component of the translocase similar to J-type cochaperones (Schneider, H.C. et al. 1994). Studies have shown that proteins entering the mitochondria are bound in the PBD of Ssc1, though the mechanism for subsequent import into the matrix remains unclear (Scherer, P.E. et al. 1990). Additionally, Ssc1 is required for the folding and assembly of protein complexes encoded in the mitochondrial DNA, as demonstrated for the ATP synthase subunit ATPase9. Using a temperature-sensitive Ssc1 mutant and a \(^{35}\text{S}\)-methionine pulse-chase method, ATPase9 was not detected in the higher molecular weight oligomers after shifting to a non-permissive temperatures indicating that oligomer assembly in the mitochondria requires the activity of Ssc1 (Hermann, J.M. et al. 1994).

Sso1 is present at lower levels in yeast mitochondria and is not essential like Ssc1 (Voisine, C. et al. 2000). Sso1 is thought to regulate the kinetics of iron-sulfur cluster biogenesis. Deletion and mutation of Sso1 has been shown to cause cold sensitivity, decreased levels of mitochondrial iron-sulfur proteins, defects in respiration, accumulation of iron in the mitochondria, greater cellular iron uptake, and reduced activity of iron-sulfur enzymes (Schilke, C. et al. 1996; Lutz, T. et al. 2001; Knight, S.A. et al., 1998; Schilke, C., et al. 1999; Garland, S.A. et al. 1999). While Sso1 appears to have a specialized function, overexpression of Ssc1 appears to be capable of rescuing the impairment in iron-sulfur cluster biogenesis (Schilke, C. et al. 2006).

1.3 Cochaperone regulation of hsp70 proteins

hsp70 chaperones require multiple J-type cochaperones to perform their functions. Assistance in the translocation of proteins across the inner mitochondrial membrane by
Ssc1 requires PAM18, a J-type cochaperone (D'Silva, P.D. et al. 2003). In addition, the folding of imported proteins by Ssc1 also requires the J-type cochaperone, Mdj1, a homolog of _E. coli_ DnaJ (Hermann, J.M. et al. 1994; Rowley, N. et al. 1994). Deletion of Mdj1 led to protein aggregation and misfolding but did not impair protein import (Prip-Buus, C. et al. 1996). Furthermore, Ssq1 relies on the J-type cochaperone Jac1 (designated HscB in bacteria and metazoans) for iron-sulfur cluster biogenesis (Lutz, T. et al. 2001).

As illustrated in Figure 1.2, J-type cochaperones promote substrate binding to their cognate chaperones by synergistically simulating ATP hydrolysis by mthsp70 and formation of the high peptide affinity state (Laufen, T. et al. 1999). J-type cochaperones interact with the ATPase domain of the chaperone through the conserved J-domain and with the substrate polypeptide through the C-terminal substrate binding domain (Wall, D. et al. 1994). ATP hydrolysis is synergistically stimulated by the binding of the J-type cochaperone and the substrate protein by >1000-fold (Liberek, K. et al. 1991).

The Ssc1 and Ssq1 reaction cycles are also regulated by a nucleotide exchange factor Mge1 (see Figure 1.2), a homolog of the _E. coli_ GrpE (Miao, B. et al. 1997; Dutkiewicz, R. et al. 2003). Like J-type proteins, GrpE binds to the ATPase domain of DnaK. GrpE initiates a conformational change which opens the deep nucleotide cleft and allows efficient exchange of ADP for ATP to restart the reaction cycle (Harrison, C.J. et al. 1997; Brehmer, D. et al. 2004).

1.4 Human mitochondrial hsp70

In mitochondria, only a single hsp70 has been identified (mtHps70). This protein is predicted to perform all the functions carried out by the yeast Ssc1 and Ssq1 homologs
including protein translocation, protein folding, and iron-sulfur cluster biogenesis (Schilke, B. et al. 2006). Human mthsp70 is nuclear encoded and is thought to have cytosolic functions in addition to its essential mitochondrial activities (reviewed in Kaul, S.C. et al. 2007). A recent study showed that mthsp70 association with p53 inhibits nuclear translocation, transcriptional activation, and control of the duplication of the centrosome, the microtubule organizing center, by p53 (Ma, Z. et al. 2006).

Although there had been extensive research examining the localization, expression levels, and in vivo functions of human mthsp70 (reviewed in Kaul, S.C. et al. 2007), there has been little work examining the in vitro structure and function of this protein. This has largely stemmed from out inability to generate sufficient recombinant mthsp70 for structure-function relationship studies. Like Ssc1 and Ssq1, mthsp70 cannot be produced in large quantities using heterologous expression systems, such as E. coli.

1.5 hsp70 escort protein (Hep1)

Mitochondrial hsp70 chaperones are responsible for preventing the off-pathway misfolding reactions of other proteins. However, they do not appear to be capable of folding by themselves and require the assistance of hsp70 escort proteins to prevent their own aggregation (Sichting, M. et al. 2005). Escort proteins were initially identified as essential proteins in a S. cerevisiae genome screen of deletion mutants (Winzeler, E.A. et al. 1999), and the single yeast hsp70 escort protein was designated Hep1. Later, Hep1 (also called Zim17 and Tim15) was identified in a BLAST analysis of the S. cerevisiae genome using the zinc-finger motif of bacterial DnaJ, and homologs were readily identified in other eukaryotes (Burri, L. et al. 2004). The functional role of two the conserved cysteines in the zinc-finger motif, C75 and C100, was examined and
determined to be necessary for Hep1 function and therefore cell survival (Yamamoto, H. et al. 2005). Figure 1.3 shows a sequence alignment for Hep1 proteins in eukaryotes highlighting the conserved residues including the four cysteines of the zinc-finger motif.

Hep1 was also found in a screen for mthsp70 binding partners and identified using mass spectroscopy (Sichting, M. et al. 2005). In this study, Hep1 was noted to have a heat shock element in the 5'-untranslated region upstream suggesting that it may be a true heat shock protein and provide stability to its binding partner, mthsp70 (Sichting, M. et al. 2005). Hep1-GFP fusion and submitochondrial fractionation and immunoblot analysis further demonstrated that Hep1 is localized to the mitochondrial matrix (Burri, L. et al. 2004; Sanjuan-Szklarz, L.K. et al. 2005; Sichting, M. et al. 2005).

Using Hep1 deletion mutants harboring inducible single-copy plasmids encoding Hep1 to selectively reduce Hep1 expression, the import of particular proteins by the different TIM complexes was quantified. Defects were found specifically in TIM23-assisted protein import (Burri, L. et al. 2004; Yamamoto, H. et al. 2005; Sichting, M. et al. 2005). Although participation in the translocase machinery was initially reported by Burri et al. as the function of Hep1, further research has indicated that this is a secondary effect (Burri, L. et al. 2004; Sichting, M. et al. 2005). Rescue of an Ssc1 mutant defect in the PBD by Hep1 overexpression indicated a genetic interaction between mthsp70 and Hep1 (Yamamoto, H. et al. 2005). Subsequent in vitro analysis using a pull-down assay with mthsp70 as bait co-purified with Hep1 and vice versa (Yamamoto, H. et al. 2005; Sichting, M. et al. 2005). Furthermore, the aggregation of mitochondrial proteins in a different Hep1 deletion strain which was viable on fermentable medium at 24°C revealed that both Ssc1 and Ssql were recovered in the insoluble fraction (Sichting, M. et al. 2005).
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<td>S. cerevisiae</td>
<td>R1---VTVEOLMANCEQVQGQWDLED1PDS1KQDVGLKRYANNENASQLHPSQK</td>
<td>174</td>
</tr>
</tbody>
</table>

Figure 1.3. Sequence alignment of Hep1 from eukaryotes. Sequence alignment of eukaryotic escort proteins. Positions having identical residues are indicated by (*), highly similar residues are indicated by (.), and weakly similar residues are indicated by (.)
Additional time-dependent experiments found mthsp70 aggregation to be the primary effect of Hep1 depletion as it occurred before other noted effects (Sanjuan-Szklarz, L.K. et al. 2005). The TIM23 complex requires mthsp70 to assist in the translocation of nascent polypeptides entering the mitochondria (Rassow, J. et al. 1994). Therefore, aggregation of mthsp70 as a result of reduced Hep1 expression is thought to produce a negative secondary effect on protein import, rather than a direct effect.

Hep1 appears to be sufficient to maintain yeast mitochondrial chaperones in a soluble conformational state. *S. cerevisiae* Ssc1 was not soluble when overexpressed alone in *E. coli*, although co-expression with *S. cerevisiae* Hep1 produced a soluble mthsp70 which was then able to be purified without Hep1 (Sichting, M. et al. 2005). A recent study with *Chlamydomonas* Hep2, a chloroplast Hep1 homolog, also showed that this class of escort proteins promote the solubility of their cognate chaperones in heterologous expression systems (Willmund, F. et al. 2008).

Hep1 depletion resulted in additional defects consistent with the loss of hsp70 functions. Aconitase, an iron-sulfur cluster protein found in the mitochondrial matrix, was quantified and reduced expression level and activity along with reduced succinate dehydrogenase activity after depletion of Hep1 suggested that mthsp70 aggregation led to this secondary effect (Sichting, M. et al. 2005). The aggregation of Ssq1 indicates that the observed decrease in iron-sulfur cluster protein expression and enzyme activity are a secondary effect of Hep1 depletion (Sichting, M. et al. 2005; Sanjuan-Szklarz, L.K. et al. 2005). Moreover, the general tubular mitochondrial morphology is lost when Hep1 expression is eliminated (Sanjuan-Szklarz, L.K. et al. 2005).
An NMR solution structure for the core domain of Hep1 (residues 64-159) has recently been solved, and the stick and ribbon diagram is shown in Figure 1.4. The structure suggests that Hep1 is an L-shaped molecule with a highly structured core that requires zinc for structural stability (Momose, T. et al. 2007). Additional Hep1 mutant screening using a yeast Hep1 deletion strain indicated that Arg 106 and His 107, Asp 111, and the loop from residues 133 to 137 are necessary to maintain cell viability (Momose, T. et al. 2007).

1.6 The small J-type cochaperone HscB

Previous studies have shown that HscB is a J-type co-chaperone which targets the substrate protein IscU, the Fe-S cluster scaffold protein, to the hsp70 chaperones and regulates the transfer of Fe-S clusters from IscU to apo-acceptor proteins (Chandramouli, K. and M.K. Johnson, 2006). HscB-type proteins are found in aerobic bacteria, fungi, and metazoans. However, our understanding of the structure and function of HscB has come primarily from studies with bacteria and yeast. In eukaryotes, HscB homologs are nuclear encoded and translocated to the mitochondria, where they are thought to have their primary functions. Figure 1.5 shows a cartoon of the role that HscB proteins play in iron-sulfur cluster biogenesis in the mitochondria.

Human HscB has an N-terminal domain (76 residues) not found in *E. coli* or *S. cerevisiae* HscB homologs, which contains a tetracysteine motif commonly found in metal binding proteins. Many multicellular organisms have homologs which contain such Cys-rich motifs, and four bacteria have HscB homologs with this motif. Figure 1.6 shows a sequence alignment of human HscB and other homologs which contain the N-terminal Cys-rich domain. This domain may serve as an additional regulator of iron-
Figure 1.4. Core domain of Hep1 NMR solution structure. Ribbon diagram of Hep1[PBD 2E2Z] illustrating the secondary structure and possible zinc coordination (Momose, T. et al. 2007). The proposed zinc coordinating cysteines are shown in blue.
Figure 1.5. Cartoon schematic for chaperone assisted iron-sulfur cluster biogenesis.

*Clockwise from top left,* First, ATP binding to hsp70 leads to a low affinity state for the substrate IscU. Second, HscB recruits the substrate protein IscU with Fe-S cluster bound to the chaperone. Third, ATP hydrolysis converts the complex to the high affinity state for substrate protein and transfers the cluster from IscU to the apo-acceptor protein. Finally, nucleotide and substrate protein (IscU) exchange restart the cycle.
**HscB domain structure**

<table>
<thead>
<tr>
<th>Cys-rich</th>
<th>J-domain</th>
<th>C-terminal domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unknown function</td>
<td>Binds HscA</td>
<td>Binds IscA</td>
</tr>
</tbody>
</table>

**HscB orthologs from mammals**

- *H. sapiens* (Hs): MWRGRAGALLRVGWFPVTGPRRPLSCDAASQAGSNYPCWGC3GPWGPGREDFFCPQALQPDPTRDYSF
- *Mus musculus* (Mm): WGRGRALLGVEWRLAGFLRGLRRLSNAAC-GKSIAPCCWNLQCHAREAGGDEFFCHCRAPQPDPTRDYSF
- *Rattus norvegicus* (Rn): WGRGARASSLKVVEWRLAGFLRGRSLSSLNAAAS-GKSIEPCWNCRGRMAGRGRGDEFFCHCRAPQPDPTRDYSF
- *Pongo troglodytes* (Pt): MWRGRGALLRVGWFPVTGPRRPLSCDAASQAGSNYPCWGC3GPWGPGREDFFCPQALQPDPTRDYSF
- *Macaca mulatta* (Mm): MWRGRGALLRVGWFPVTGPRRPLSCDAASQAGSNYPCWGC3GPWGPGREDFFCPQALQPDPTRDYSF
- *Canis familiaris* (Cf): MWGRTGALLRVGWLPAGALGRRPLSCDAASAGGSCGCGCGGPPGPGFTRGDFFCPQALQPDPTRDYSF
- *Bos taurus* (Bt): MWGRTGALLRVGWLPAGALGRRPLSCDAASAGGSCGCGCGGPPGPGFTRGDFFCPQALQPDPTRDYSF
- *Gallus gallus* (Gy): MWGRTGALLRVGWLPAGALGRRPLSCDAASAGGSCGCGCGGPPGPGFTRGDFFCPQALQPDPTRDYSF
- *T. gondii* (Tg): MWGRTGALLRVGWLPAGALGRRPLSCDAASAGGSCGCGCGGPPGPGFTRGDFFCPQALQPDPTRDYSF

**Cys-rich HscB orthologs from prokaryotes**

- *M. tuberculosis* (Mt): ALApAATIYSSHCGPVATRALFCSGAVPGPG-NIDHFSR
- *A. baumannii* (Ab): AARSHSQKAEQQPFPFCSKIQPVGRTEDYFSR
- *A. baumannii* (Ab): MAFRTYCSAIPVLOGSARRTVPWWSR--DRBAAHFCQNCSSWQPAP--PTDYSF
- *L. lactis* (Lc): MGEHTRHSQGASSLOKNKGL-TLEADDLTVKQPFPKEDROYFDI
- *E. coli* (Ec): MDYFTL
- *S. cerevisiae* (Sc): MLKYLQORRFTSTFYELFFKF

**Figure 1.6. HscB domain structure and multiple sequence alignment of the N-terminus of HscB.** *H. sapiens*, *M. musculus*, *R. norvegicus*, *P. troglodytes*, *M. mulatta*, *C. familiaris*, *B. Taurus*, *G. gallus*, *T. guttata*, *M. magneticum*, *A. dehalogenans*, *Acidobacteria*, *L. ferrooxidans*, *E. coli* and *S. cerevisiae* HscB (blue). Sequence identity (*) and similarity (. or :). Cysteines are highlighted in green.
sulfur cluster biogenesis in these organisms. The function of these domains has not been established.

1.7 Significance

Hep1 is essential for maintaining mthsp70 solubility in S. cerevisiae (Sichting, M. et al. 2005). However, there has been little study of its metal content, substrate specificity, and thermostability. In addition, it remains unclear whether it functions by stabilizing a native or non-native conformational state of its chaperone substrates. The studies described herein provide direct insight into Hep1 cofactor requirements, substrate specificity and thermostability.

All eukaryotes appear to contain an HscB cochaperone, but some eukaryotes and a handful of prokaryotes have an HscB cochaperone that contains an extra cysteine-rich N-terminal domain. Based on homology to rubredoxins (Perry, A. et al. 2001) and preliminary data described herein this domain is predicted to bind iron. To date, there have been no reports on the structure or function of these long HscB homologs, and it is unclear why some prokaryotes and eukaryotes have evolved cochaperones with this domain. My studies with M. magneticum and human HscB proteins provide insight into the structure of this cofactor and its possible role in providing protein level regulation of chaperone-assisted Fe/S-cluster biogenesis.

Human mthsp70 is a vital component of mitochondrial biogenesis and cellular viability. Although mthsp70 is primarily localized to the mitochondria, cellular studies have also identified it in other subcellular locations including the endoplasmic reticulum, vesicles in the cytosol, plasma membrane, and cytosol (Domanico, S.Z. et al. 1993; Singh, B. et al. 1997; Soltys, B.J. and R.S. Gupta, 1999; Ran, Q. et al. 2000). Studies
have linked these populations of mthsp70 with additional protective roles and functions. In fact, increased expression of mthsp70 has been correlated with human carcinogenesis (Wadhwa, R. et al. 2006). Brain tumor cells have been shown to have elevated mthsp70 expression relative to normal brain cells (Takano, S. et al. 1997). A correlation between mthsp70 expression and colorectal cancer prognosis has also been found (Dundas, S. R. et al. 2004). Furthermore, mutation of the zebrafish homolog (HspA9b) of mthsp70 exhibited a phenotype similar to myelodysplastic syndrome, a bone marrow stem cell blood disorder with ineffective blood cell production that often progresses to leukemia (Craven, S.E. et al. 2005). Additionally, the lifespan of fibroblasts was extended by overexpression of mthsp70 (Kaul, S.C. et al. 2000). Interestingly, a complex of mthsp70 and p53 has been localized to duplicated centrosome during late G1, S, and G2 thus indicating a role in cell cycle regulation (Ma, Z. et al. 2006). Thus, an explanation for the role that mthsp70 has in cancer development could be that interactions of mthsp70 with p53 lead to inactivation of p53 and subsequent uncontrolled proliferation of the cell causing cancer. It remains unclear whether the human Hep1 homolog will be required to maintain mthsp70 in a conformational state that is competent for p53 binding.

Furthermore, mthsp70 has been implicated in diabetes (Muranyi, M. et al. 2005) and other neurodegenerative diseases associated with aging including Alzheimer’s disease (AD), Parkinson’s disease (PD), Huntington’s disease (HD), and others. One study demonstrated that PD patients have decreased expression of mthsp70 (Jin, J. et al. 2006). Another study linked familial PD caused by the DJ-1 gene to mthsp70 association (Li, H.M. et al. 2005). mthsp70 was also shown to bind to α–synuclein, another contributor in PD pathogenesis (Jin, J. et al. 2007). Intriguingly, during normal nematode
aging, mthsp70 expression decreased and overexpression of mthsp70 increased worm lifespan (Yokoyama, H. et al. 2002).

As many studies have indicated, mthsp70 is a target for cancer and aging therapies. This research describes a method for producing large quantities of human mthsp70 which can be used for future research including potential drug targeting against the aforementioned diseases.
CHAPTER 2

MATERIALS AND METHODS

2.1 Yeast Hep1 plasmid design, construction, and generation of mutants

The plasmid pET28b-ScHep1 was generated by PCR amplifying the Hep1 gene from *S. cerevisiae* genomic DNA (Invitrogen) and cloning this gene into the pET28b(+) vector (Novagen) using unique *NcoI* and *HindIII* restriction sites. The mitochondrial targeting sequence was removed from Hep1 (Sichting, M. et al. 2005) and mthsp70 (Bhattacharyya, T. et al. 1995) a subsequently, a methionine was inserted at the N-terminus to initiate transcription. Additionally, a glycine was inserted after the initiating methionine to facilitate cloning of Hep1. The plasmid pET28b-ScHep1-His was generated using site-directed mutagenesis of the pET28b-ScHep1 vector to eliminate the stop codon for the gene and incorporate the hexahistidine tag (KLAAAALEHHHHHH) already engineered into the pET28b(+) vector (Novagen). The plasmids pET28b-ScHep1-C75S, pET28b-ScHep1-C78S, pET28b-ScHep1-C100S, and pET28b-ScHep1-C103S were generated by site-directed mutagenesis of the pET28b-ScHep1 vector to change the codon of the corresponding residue from cysteine to serine. Table 2.1 outlines these expression constructs which have been used for the following studies. Each of these vectors was sequence verified to ensure that mutations were not introduced.

2.2 Human mthsp70 plasmid design, construction, and generation of mutants

The plasmid pET21d-mthsp70 was generated by PCR amplifying the human mthsp70 gene from pOTB7-hsp70 (Invitrogen - Accession BC000478) and cloning this gene into the pET21d(+) vector (Novagen) using *NcoI* and *NsiI* restriction sites. The
<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Protein Product</th>
<th>Sequenced</th>
<th>Construction Strategy</th>
</tr>
</thead>
<tbody>
<tr>
<td>pET21d-mthsp70</td>
<td>human mthsp70 (residues 47-679)</td>
<td>Yes</td>
<td>Subcloned from pOTB7-mthsp70 (Invitrogen)</td>
</tr>
<tr>
<td>pET21d-mthsp70-His</td>
<td>human mthsp70, His-tagged (47-679)</td>
<td>Partially</td>
<td>Subcloned from pOTB7-mthsp70 (Invitrogen)</td>
</tr>
<tr>
<td>pET21d-mthsp70(47-385)</td>
<td>human mthsp70 (residues 47-385)</td>
<td>Yes</td>
<td>Subcloned from pET21d-mthsp70</td>
</tr>
<tr>
<td>pET21d-MmHscB</td>
<td>M. magneticum HscB</td>
<td>Yes</td>
<td>Cloned from M. magneticum genomic DNA</td>
</tr>
<tr>
<td>pET21d-MmHscB-His</td>
<td>M. magneticum HscB, His-tagged</td>
<td>Yes</td>
<td>Cloned from M. magneticum genomic DNA</td>
</tr>
<tr>
<td>pET21d-HsHscB</td>
<td>Human HscB (1-236)</td>
<td>Yes</td>
<td>Subcloned from pTRC-HscB (from Vickers @ UC-I)</td>
</tr>
<tr>
<td>pET21d-HsHscB-FL</td>
<td>human HscB (1-236)</td>
<td>Yes</td>
<td>Synthesized from primers</td>
</tr>
<tr>
<td>pET21d-HsHscB-FL-His</td>
<td>human HscB (1-236), His</td>
<td>Yes</td>
<td>Quikchange pET21d-HsHscB(1-236)</td>
</tr>
<tr>
<td>pEGFP-N1-HsHscB-FL</td>
<td>human HscB (1-236), GFP fusion</td>
<td>Yes</td>
<td>Subcloned from pET21d-HsHscB(1-236)</td>
</tr>
<tr>
<td>pET28b-SchEp1</td>
<td>SchEp1 (48-174)</td>
<td>Yes</td>
<td>Cloned from S. cerevisiae genomic DNA</td>
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<tr>
<td>pET28b-SchEp1-His</td>
<td>SchEp1 (48-174), His</td>
<td>Yes</td>
<td>Quikchange pET28b-SchEp1 no stop</td>
</tr>
<tr>
<td>pET28b-SchEp1-C30S</td>
<td>SchEp1 (48-174) with C30S mutation</td>
<td>Yes</td>
<td>Quikchange pET28b-SchEp1 C30S</td>
</tr>
<tr>
<td>pET28b-SchEp1-C33S</td>
<td>SchEp1 (48-174) with C33S mutation</td>
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<td>Quikchange pET28b-SchEp1 C33S</td>
</tr>
<tr>
<td>pET28b-SchEp1-C555S</td>
<td>SchEp1 (48-174) with C55S mutation</td>
<td>Yes</td>
<td>Quikchange pET28b-SchEp1 C55S</td>
</tr>
<tr>
<td>pET28b-SchEp1-C588S</td>
<td>SchEp1 (48-174) with C58S mutation</td>
<td>Yes</td>
<td>Quikchange pET28b-SchEp1 C58S</td>
</tr>
</tbody>
</table>
plasmids pET21d-mthsp70 ATPase domain was generated by site-directed mutagenesis to incorporate a stop codon after residue 385.

2.3 Human HscB plasmid design and construction

The plasmid pET21d-HsHscB(71-236) was generated by PCR amplifying the HsHscB gene from pTRC-HscB (kindly provided by Larry Vickery at UC Irvine) and cloning this gene into the pET21d(+) vector (Novagen) using NcoI and HindIII restriction sites. This plasmid contains an N-terminal initiating methionine insertion as the predicted mitochondrial signal sequence was removed. The plasmid pET21d-HsHscB(1-236) was generated by subcloning the C-terminal fragment from pET21d-HsHscB(71-236) and engineered primer annealing and ligation to generate the N-terminal fragment from the corresponding amino acid residues 1-70. The plasmid pET21d-HsHscB(1-236)-His was generated using site-directed mutagenesis of the untagged parent vector to eliminate the stop codon for the gene and incorporate the hexahistidine tag (KLAAALEHHHHHH) already engineered into the pET21d(+) vector (Novagen). The plasmid pEGFP-N1-HsHscB(1-236) was generated by subcloning the gene from pET21d-HsHscB(1-236) into and cloning this gene into the pEGFP-N1 vector (Clonetech) using BamHI and HindIII restriction sites. The full-length HsHscB clones (1-236) contain an inserted glycine following the initiating methionine for cloning purposes.

2.4 Magnetospirillum magneticum HscB plasmid design and construction

The plasmids pET21d-MmHscB, pET21d-MmHscB-His, pET21d-mmHscB(39-208), and pET21d-MmHscB(39-208)-His were generated by PCR amplifying the MmHscB gene from genomic DNA (kindly provided by Arash Komeli at UC Berkley) and cloning into the pET21d(+) vector (Novagen) using NcoI/PciI and HindIII restriction
sites. The tagged version of each clone incorporates the hexahistidine tag (KLAAALEHHHHHH) already engineered into the pET21d(+) vector (Novagen). The full-length constructs contain an inserted leucine after the initiating methionine for cloning purposes. The truncated constructs contain an inserted methionine and leucine as the predicted mitochondrial signal sequence was removed.

2.5 Protein expression

Because the mthsp70 and various Hep1 plasmids were not codon optimized for expression in *E. coli*, they were transformed into Rosetta2 *E. coli*, a cell line containing the pRARE vector which produces seven rare tRNAs to enhance expression of heterologous proteins. Transformed cells were grown at 37°C in Luria Broth containing 50 µg/mL ampicillin, kanamycin, or both for mthsp70 alone, Hep1 alone, or mthsp70 and Hep1, respectively. Cells were induced at OD$_{600}$ = 1.0 with a final concentration of 0.1 mM β-D-1-isopropylthiogalactopyranoside (IPTG) and the temperature was reduced to 25°C for six hours. Cells were harvested by centrifugation at 5,000 rpm for 30 minutes and lysed in 20 mL/L of lysis buffer (10 mM Tris pH 8.0, 1 mM DTT, 1 mM MgCl$_2$, 2U/mL Dnase1, 40 mg/L lysozyme) for 40 minutes at 4°C followed by freezing to -80°C.

Because the HscB plasmids were also not codon optimized for expression in *E. coli*, they were transformed into Rosetta2 *E. coli*, a cell line containing the pRARE vector which produces seven rare tRNAs to enhance expression of heterologous proteins. Transformed cells were grown at 37°C in Luria Broth containing 50 µg/mL ampicillin. Cells were induced at OD$_{600}$ = 1.0 with a final concentration of 0.1 mM β-D-1-isopropylthiogalactopyranoside (IPTG) and the temperature was reduced to 25°C for six hours. Cells were harvested by centrifugation at 5,000 rpm for 30 minutes and lysed in
20 mL/L of lysis buffer (10 mM Tris pH 8.0, 1 mM DTT, 1 mM MgCl₂, 2U/mL DnaseI, 40 mg/L lysozyme) for 40 minutes at 4°C followed by freezing to -80°C.

2.6 Expression of Hep1 in M9 minimal media with zinc supplementation

Cells transformed with pET28b-Hep1 were grown at 37°C in M9 minimal media containing 50 μg/mL kanamycin supplemented with 0, 0.1 or 1.0 mM ZnCl₂. Cells were induced at OD₆₀₀ = 1.0 with a final concentration of 0.1 mM IPTG and the temperature was reduced to 25°C for six hours. Cells were harvested, lysed, and frozen as outlined for the standard procedure.

2.7 SDS-PAGE analysis of yeast Hep1 and human mthsp70 protein solubility

Frozen cell lysate of cells expressing the indicated proteins were thawed and centrifuged at 20,000 rpm for 60 minutes to remove cell debris. The insoluble pellet was the resuspended in TED buffer and the protein concentrations for the total cell lysate, soluble fraction, and insoluble fraction were determined using the Bio-Rad Protein Assay (BioRad). For each sample, 15 μg of protein was loaded for SDS-PAGE analysis.

2.8 Purification of S. cerevisiae His-tagged Hep1

Frozen cell lysate of Hep1-His was thawed and centrifuged at 20,000 rpm for 60 minutes to remove cell debris. This was followed by syringe filtering the supernatant with a 0.22 μM filter. The filtered soluble fraction was diluted 1:1 with binding buffer (20 mM Imidazole, 20 mM KH₂PO₄, 0.5 M NaCl, 1 mM DTT) and loaded onto nickel nitrotriacetic acid (Ni-NTA) resin (Qiagen) which had been equilibrated with two column volumes of binding buffer. The column was then washed to remove non-specific protein interacting with the resin with two additional column volumes of binding buffer. The Hep1-His was then eluted with 500 mM Imidazole binding buffer. The eluted protein
was then dialyzed against 10 mM Tris pH 8.0 for 20 hours. Protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad). This preparation gave a typical yield of ~25 mg/L.

2.9 Purification of human mthsp70

Frozen cell lysate from cells co-overexpressing mthsp70 AND Hep1 was thawed and centrifuged at 20,000 rpm for 60 minutes to remove cell debris. This was followed by syringe filtering the supernatant with a 0.22 μM filter. The filtered soluble fraction was loaded onto a DE52 resin column (Whatman) which had been equilibrated with two column volumes of TED (50 mM Tris, pH 8.0, 0.5 mM EDTA, 1 mM DTT) buffer. The column was then washed to remove non-specific protein interacting with the resin with two additional column volumes of binding buffer. The mthsp70 was then eluted with a gradient to 200 mM NaCl TED buffer. Homogenous fractions were pooled and ammonium sulfate precipitated at 50% saturation. The protein was then pelleted by centrifugation at 20,000 rpm for 10 min. The pellet was resuspended in TED buffer and dialyzed against TED to remove remaining ammonium sulfate. Protein concentration was determined using the BioRad Protein Concentration Assay (Bio-Rad). This preparation gave a typical yield of ~25 mg/L.

2.10 Purification of human HscB(71-236)

Frozen cell lysate of human HscB was thawed and centrifuged at 20,000 rpm for 60 minutes to remove cell debris. This was followed by syringe filtering the supernatant with a 0.22 μM filter. The filtered soluble fraction was diluted 1:1 with HED buffer (10 mM HEPES pH 7.5, 1 mM EDTA, 1 mM DTT) and loaded onto a DE52 resin column (Whatman) which had been equilibrated with two column volumes of HED buffer. The
column was then washed to remove non-specific protein interacting with the resin with two additional column volumes of HED buffer. The protein was then eluted with a gradient to 500 mM NaCl HED buffer. Fractions of pure HscB were pooled and the eluted protein was then dialyzed against HED buffer for 16 hours. Protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad). This preparation gave a typical yield of \(\sim 25\) mg/L.

2.11 Purification of *Magnetospirillum magneticum* HscB

Frozen cell lysate of MmHscB-His was thawed and centrifuged at 20,000 rpm for 60 minutes to remove cell debris. This was followed by syringe filtering the supernatant with a 0.22 \(\mu\)M filter. The filtered soluble fraction was diluted 1:1 with binding buffer (20 mM Imidazole, 20 mM \(\text{KH}_2\text{PO}_4\), 0.5 M NaCl, 1 mM DTT) and loaded onto nickel nitrotriacetic acid (Ni-NTA) resin (Qiagen) which had been equilibrated with two column volumes of binding buffer. The column was then washed to remove non-specific protein interacting with the resin with two additional column volumes of binding buffer. The MmHscB-His was then eluted with 500 mM Imidazole binding buffer. The eluted protein was then dialyzed against 10 mM Tris pH 8.0 for 20 hours or flash frozen in a dry ice/ethanol bath and stored in liquid nitrogen. Protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad). This preparation gave a typical yield of \(\sim 25\) mg/L.

2.12 Zinc determination

A standard curve for the absorbance change at 500 nm was generated for the PAR₂\((\text{Zn}^{2+})\) (4-(2-pyridylazo)-resorcinol) complex using 2.5, 5, 7.5, 10, 12.5, and 15 \(\mu\)M zinc chloride stock solutions. 5 \(\mu\)M Hep1 was then reacted with excess methyl
methanethiosulfonate (MMTS) and PAR in 50 mM Hepes, pH 7.5 and plotted against the standard curve to determine the stoichiometry. All absorbance readings were baseline corrected for buffer (MMTS, PAR and Hepes) absorbance.

2.13 UV-VIS absorbance spectroscopy

UV-VIS absorbance spectra were obtained using a Cary 50 spectrophotometer at room temperature. A spectrum of purified mthsp70 was taken using 1.7 μM mthsp70 in TED buffer baseline corrected for the buffer absorbance. A spectrum of purified MmHscB-His was taken in 500 mM Imidazole binding buffer baseline corrected for the buffer absorbance.

2.14 Circular dichroism spectroscopy

Circular dichroism spectra were obtained using a Jasco J-810 spectropolarimeter using a 0.1 cm pathlength cuvette. 6 μM Hep1 was dialyzed into 10 mM Tris, pH 8.0. A second 6 μM Hep1 was denatured by adding GuHCl to a final concentration of 5 M GuHCl. 6 μM mthsp70 was dialyzed into 10 mM Tris pH 8.0, 1 mM EDTA, 1 mM DTT. 6 μM Hep1 and 3 μM mthsp70 were dialyzed into TED buffer. Reference spectra for the buffer were subtracted from the average of 16 spectra accumulations. Ellipticity was monitored from 190 nm to 260 nm. Thermal denaturation curves were monitored at 222 nm with scan rates of 0.5°C/min and 1°C/min from 20-85°C in 10 mM K₂HPO₄. Protein unfolding was irreversible for all samples. The circular dichroism spectrum for 6 μM human HscB(71-236) in HED buffer was obtained from 200 to 260 nm subtracting reference spectra for the buffer.

2.15 Analytical size exclusion chromatography
The apparent molecular mass of human HscB(71-236) was measured by size exclusion chromatography using an S200 (Whatman) column with standards in HED buffer. The standards used were amylase (200 kDa), alcohol dehydrogenase (158 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa).

2.16 Cell culture

Human embryonic kidney (HEK293) cells were cultured in Dulbecco’s modified Eagle’s medium (Fisher) with 10% bovine calf serum at 37°C with 90% humidity and 5% CO₂. Cells were transfected at ~70% confluency per the Fugene6 manufacturer’s transfection instructions (Roche). Cell staining was performed with 45 minutes in 100 nM Mitotracker Red (Molecular Probes) with PBS buffer. Cells were washed in PBS buffer before imaging with a Zeiss LSM-510 confocal fluorescence microscope.
CHAPTER 3

HEP1 AND mtHSP70 RESULTS

3.1 *Saccharomyces cerevisiae* HEP1 cysteines are required for solubility

Previous studies have demonstrated that two of the four conserved cysteines are necessary for maintaining HEP1 function (Yamamoto, H. et al. 2005) implicating a possible role for these cysteine residues in maintaining HEP1 solubility. To explore this possibility, *S. cerevisiae* HEP1 without its N-terminal mitochondrial signal sequence (Sichting, M. et al. 2005) was cloned into pET28b(+), an IPTG inducible expression plasmid. Each of the four conserved cysteines were mutated to serines (C75S, C78S, C100S, C103S) using Quickchange site-directed mutagenesis. The solubility of HEP1 and each of these mutants was determined by heterologous overexpression in Rosetta2 *E. coli*.

Figure 3.1 shows the expression of HEP1 and the four cysteine mutants. Expression of all HEP1 variants was noted as a significant band with an apparent molecular weight (~18 kDa) that is greater than the predicted molecular weight (14.6 kDa). Analysis of the soluble and insoluble fractions of cells expressing HEP1 indicated that HEP1 was predominantly found in the soluble fraction. In contrast, the C75S, C78S, C100S, and C103S HEP1 mutants were not detected in the soluble fraction, although their expression was consistent with that for native HEP1. This suggests that exchange of oxygen for any of the sulfur atoms in the conserved cysteine residues eliminates HEP1 solubility. Since the cysteines in HEP1 are thought to coordinate zinc, this also suggests that HEP1 requires bound zinc to maintain its solubility.

3.2 Zinc content of HEP1
<table>
<thead>
<tr>
<th></th>
<th>Hep1</th>
<th>C75S</th>
<th>C78S</th>
<th>C100S</th>
<th>C103S</th>
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**Figure 3.1. Hep1 cysteines are required for solubility.** Lane 1, molecular weight standards; Lanes 2-4, contain total protein, soluble lysate, and pellet fractions from cells harboring pET28b-Hep1; Lanes 5-7, contain total protein, soluble lysate, and pellet fractions from cells harboring pET28b-Hep1-C75S; Lanes 8-10, contain total protein, soluble lysate, and pellet fractions from cells harboring pET28b-Hep1-C78S; Lanes 11-13, contain total protein, soluble lysate, and pellet fractions from cells harboring pET28b-Hep1-C100S; Lanes 14-16, contain total protein, soluble lysate, and pellet fractions from cells harboring pET28b-Hep1-C103S. For each sample, 15 µg of protein was loaded for samples of total protein (T) from whole cells and soluble (S) and pellet (P) fractions after lysis and separation by centrifugation. Protein concentrations were determined using the Bio-Rad Protein Assay.
To examine whether the four conserved cysteines in Hep1 constitute a metal-binding motif as predicted from NMR studies (Momose, T. et al. 2007), the solubility of Hep1 was examined when expressed in Rosetta2 E. coli grown in M9 minimal media containing or lacking supplemental zinc chloride. Figure 3.2 shows that IPTG-induced expression of Hep1 in minimal media led to a major band similar to that observed when E. coli was grown in LB medium. However, Hep1 expression in minimal media lacking supplemental zinc yielded insoluble protein, as well as Hep1 expressed in minimal media supplemented with 0.1 mM ZnCl₂. In contrast, when Hep1 was expressed in minimal media containing 1 mM ZnCl₂, approximately half of the protein was soluble, similar to that observed upon expression in rich LB media.

To obtain recombinant Hep1 for in vitro analysis, I created an IPTG-controlled expression plasmid for producing Hep1 with a C-terminal (His)₆ tag. His-tagged Hep1 was expressed in Rosetta2 E. coli grown in LB medium and an SDS-PAGE was used to evaluate its solubility. His-tagged Hep1 was largely found in the soluble fraction with a molecular weight slightly larger than observed for untagged Hep1 (see Figure 3.1). Hep1 with a C-terminal His tag was readily purified as a soluble protein using nickel talon affinity (NTA) chromatography and dialyzed against 10 mM Tris pH 8.0 for 20 hours. SDS-PAGE analysis of purified S. cerevisiae Hep1 (see Figure 3.3) indicates that Hep1 is ≥95% homogeneous.

To directly determine whether Hep1 contains bound zinc as predicted from structural studies (Momose, T. et al. 2007), inductively coupled plasma – atomic emission spectrophotometry (ICP-AES) was used to evaluate the metal content of Hep1. Purified Hep1 was determined to possess an approximately stoichiometric amount of
**Figure 3.2.** Zinc is required for soluble heterologous expression of Hep1. Lane 1, molecular weight standards; Lanes 2-4 contain total protein, soluble lysate, and pellet fractions from cells harboring pET28b-Hep1 grown in M9 media without zinc; Lanes 5-7 contain total protein, soluble lysate, and pellet fractions from cells harboring pET28b-Hep1 grown in M9 media supplemented with 0.1 mM zinc; Lanes 8-10 contain total protein, soluble lysate, and pellet fractions from cells harboring pET28b-Hep1 grown in M9 media supplemented with 1 mM zinc.
Figure 3.3. Determination of *S. cerevisiae* Hep1 purity. SDS-PAGE analysis of *S. cerevisiae* Hep1 protein purity. Lane 1, molecular weight standards; Lanes 2-4, 1, 3, and 10 μg of purified *S. cerevisiae* Hep1 was loaded for samples.
bound zinc (0.8 zinc molecules per Hep1). Figure 3.4 shows a diagram of how ICP-AES detects metals in the sample. To further determine whether it was the four conserved cysteine residues which were responsible for zinc coordination, purified Hep1 (6.6 μM) was reacted with 30-fold excess methyl methanethiosulfonate (MMTS), a sulfhydryl-reactive agent, and subsequent zinc release was monitored using 4-(2-pyridylazo)-resorcinol (PAR), a zinc chelator which displays an increase in absorbance at 500 nm upon zinc binding. An absorbance increase corresponding to one zinc molecule per Hep1 was observed indicating that all zinc is released following MMTS treatment. Figure 3.5 shows the stoichiometry of the absorbance increase following zinc release by MMTS titration.

3.3 S. cerevisiae Hep1 thermal stability

Previous studies determined that Hep1 is required for the solubility of mitochondrial hsp70-type chaperones (Sichting, M. et al. 2005), suggesting that Hep1 have evolved to withstand at high temperatures. To establish the thermostability of Hep1, the change in ellipticity at 222 nm was monitored from 20-85°C. Figure 3.6 shows the far-UV CD spectrum for His-tagged Hep1 from 190 to 260 nm. As previously reported (Momose, T. et al. 2007), this spectrum shows a minimum at 206 nm and lacks significant minima at 208 and 222 nm characteristic of α-helix and at 215 nm characteristic of β-sheet. Figure 3.7 shows the effect of temperature on the ellipticity of His-tagged Hep1 at 222 nm. Little change in ellipticity was observed as the temperature was raised from 20-70°C. The lack of measurable secondary structural changes in Hep1 implies that it is extremely thermostable with the cofactor bound to 70°C. Full spectrum scans from 190 to 260 nm at temperature intervals also indicated that there were no
Figure 3.4. Determination of *S. cerevisiae* Hep1 metal content by ICP-AES. The purified Hep1 sample was atomized by a nebulizer and introduced directly into the argon gas plasma flame and excited to ~6,000 K. Subsequent excitation of electrons and return to ground state produces characteristic emission spectra which are detected and evaluated for specific metal content (Alcock, N.W., 1995).
Figure 3.5. The stoichiometry of Hep1 to zinc is 1:1. (■), the standard curve for zinc using 2.5, 5, 7.5, 10, 12.5, and 15 μM zinc stock solutions to measure the absorbance at 500 nm following formation of the PAR$_2$Zn$^{2+}$ complex in excess PAR reagent. (○), the measured absorbance for 5 μM Hep1 in 50 mM HEPES, pH 7.5, in the presence of excess PAR reagent following displacement of zinc from the protein with excess MMTS reagent.
Figure 3.6. CD spectrum for Hep1 indicates little secondary structure. CD spectrum for (●) 6 μM Hep1 in 10 mM Tris, pH 8.0 with sixteen accumulations showing little measured ellipticity and (●) 6 μM Hep1 in 10 mM Tris, pH 8.0 and 5 M GuHCl. Inset shows the spectra for α-helix and β-sheet based on 100% secondary structure calculations.
Figure 3.7. **Hep1 is extremely thermostable.** Circular dichroism signal for 3 µM Hep1 in 10 mM Tris, pH 8.0 were acquired over a range of temperatures. Ellipticity was monitored at 222 nm with a scan rate of 1°C/min from 20-85°C in the same buffer. Protein unfolding was irreversible. A difference spectrum is shown for wavelength scans from 190 to 260 nm at 20 and 70°C.
significant changes in ellipticity at any wavelength for Hep1 from 20-70°C though higher temperatures led to aggregation of Hep1.

3.4 *S. cerevisiae* Hep1 promotes human mthsp70 solubility

To determine whether *S. cerevisiae* Hep1 can promote the folding of a non-cognate hsp70 chaperone, I investigated its effects on the solubility of a mammalian chaperone. I created an IPTG-controlled expression plasmid for producing human mthsp70 in *E. coli* (designated phsp70) and characterized the expression level and solubility of mthsp70 when expressed alone or with Hep1. Figure 3.8 shows an SDS-PAGE gel in which IPTG-induced Rosetta2 *E. coli* cells harboring phsp70 in the presence and absence of pHep1 were analyzed. For cells containing phsp70, a major band is present that migrates at an apparent molecular weight that is similar to that calculated for mthsp70 (68.9 kDa). Analysis of the soluble and insoluble fractions of lysed cells indicates that mthsp70 is predominantly detected within the insoluble fraction. Cells harboring both phsp70 and pHep1 produced the same level of mthsp70 as cells harboring only phsp70. Under these conditions, however, more than half of the mthsp70 is present in the soluble fraction, indicating that yeast Hep1 supports the production of soluble human mthsp70.

3.5 Purification of recombinant human mthsp70

Human mthsp70 was co-expressed in Rosetta2 *E. coli* with Hep1 grown in LB medium. Human mthsp70 was largely found in the soluble fraction and was purified using two sequential anion exchange chromatography, size exclusion chromatography, and dialyzed against 10 mM Tris pH 8.0, 1 mM EDTA, 1 mM DTT. Figure 3.9A shows an SDS-PAGE analysis of mthsp70 protein purity.
Figure 3.8. *S. cerevisiae* Hep1 maintains the solubility of human mthsp70. Lane 1, molecular weight standards; Lane 2, Rosetta 2 *E. coli*; Lanes 3-5 contain total protein, soluble lysate, and pellet fractions from cells harboring phsp70; Lanes 4-6 contain total protein, soluble lysate, and pellet fractions from cells harboring pHep1, Lanes 7-9 contain total protein, soluble lysate, and pellet fractions from cells harboring phsp70 and pHep1. For each sample, 15 µg of protein was loaded for samples of total protein (T) from whole cells and soluble (S) and pellet (P) fractions after lysis and separation by centrifugation. Protein concentrations were determined using the Bio-Rad Protein Assay.
Figure 3.9. Determination of human mthsp70 purity. A, SDS-PAGE analysis of human mthsp70 protein purity. Lane 1, molecular weight standards; Lanes 2-4, 1, 3, and 10 μg of purified human mthsp70 were loaded for samples. Based on these proportions, purified human mthsp70 is more than 95% pure. B, A UV-VIS spectrum of 1.7 μM human mthsp70 showing absorbance at 280 nm and not at 260 nm indicating little nucleotide contamination.
The endogenous bacterial hsp70-type chaperone, DnaK, retains nucleotide during purification (Russell, R. et al. 1998). To determine if this was the case for human mthsp70, a UV-VIS spectrum of purified human mthsp70 was examined from 240 to 340 nm. Figure 3.9B shows that purified human mthsp70 lacks a peak at 260 nm. This indicates that little nucleotide is in the sample. In addition, it suggests that our sample lacks significant contaminating endogenous DnaK, which has a molecular weight of ~70 kDa.

The secondary structure was evaluated by CD spectroscopy to determine if the overall folding of the purified mthsp70 was similar to that found for other mthsp70s. Figure 3.10 shows the far-UV CD spectrum for purified mthsp70. Using the K2D algorithm, the mthsp70 secondary structure content was estimated to be 35% α-helix, 18% β-sheet and 47% random coil (Andrade, M.A. et al. 1993). In addition, the crystal structure of the ATPase domain of bovine Hsc70 has a 38% α-helix and 30% β-sheet secondary structure content (Flaherty, K.M. et al. 1990). The crystal structure of the PBD of DnaK has a secondary structure content of 33% α-helix and 27% β-sheet (Zhu, X. et al. 1996). These percentage predictions of α-helix and β-sheet found in mthsp70 are consistent with the overall secondary structure predicted for a well-folded hsp70.

3.6 *S. cerevisiae* Hep1 does not stabilize human mthsp70

To investigate the mechanism by which Hep1 interacts with mthsp70 to promote proper folding, I examined the effects of *S. cerevisiae* Hep1 on the thermal unfolding of human mthsp70 using CD spectroscopy. In these experiments, I monitored the change in ellipticity at 222 nm as the temperature was increased from 20-85°C at a rate of 1°C/min. This wavelength was used because Hep1 lacked appreciable signal change at this
Figure 3.10. CD spectra for mthsp70. Circular dichroism spectra for 6 µM mthsp70 dialyzed into TED buffer with 16 accumulations.
wavelength (see Figure 3.7). Figure 3.11 shows the changes in ellipticity observed for a sample containing human mthsp70 alone. Under these conditions, two transitions were observed with midpoints at 48°C and 78°C. In the presence of Hep1, the midpoints for the two transition temperatures were similar (46°C and 72°C). Experiments performed using scan rates of 0.5°C/min and 1°C/min yielded different transition temperatures. The slower scan rate resulted in a shift of the midpoint transition temperatures to 45°C and 76°C. The lack of a major effect of Hep1 on mthsp70 unfolding suggests that Hep1 does not stabilize mthsp70 under conditions of thermal denaturation.

3.7 Domain requirements for mthsp70 insolubility

Previous studies on mutants of yeast mthsp70 showed that a mutation in the ATPase domain affected Hep1 interaction, whereas a mutation in the PBD did not (Sichting, M. et al. 2005). Furthermore, expression of yeast mthsp70 in *E. coli* requires Hep1 for solubility (Sichting, M. et al. 2005). Interestingly, the ATPase domain alone was insoluble in the presence or absence of Hep1 (Sichting, M. et al. 2005). However, the PBD was soluble in the absence of Hep1 (Sichting, M. et al. 2005). This data seems to suggest that Hep1 interacts with the ATPase domain but also requires the PBD. To determine if the ATPase domain of human mthsp70 is also insoluble when expressed separately, an IPTG-controlled human mthsp70-ATPase plasmid was generated. Quikchange mutagenesis was used to insert a stop codon after residue 385 in the vector pET21d-mthsp70. The solubility of the ATPase domain was then evaluated by heterologous overexpression in Rosetta2 *E. coli*.

Figure 3.12 shows the expression of human mthsp70 ATPase domain. Expression of this truncation was noted as a significant band with an apparent molecular weight
Figure 3.11. The change in ellipticity for the thermal transitions of mthsp70 and mthsp70 with Hep1. Circular dichroism thermal denaturation curves for 6 μM mthsp70 (black) and 6 μM mthsp70 with 3 μM Hep1 (gray) in TED buffer. Ellipticity was monitored at 222 nm with a scan rate of 1°C/min from 20-85°C in 10 mM K2HPO4. Protein unfolding was irreversible for all samples.
Figure 3.12. Domain requirements for mthsp70 insolubility. Lane 1, molecular weight standards; Lanes 2-3 contain total protein and soluble lysate from cells harboring pET21d-mthsp70-ATPase. For each sample, 1.5 µL was loaded for samples of total protein (T) and soluble fractions (S) after lysis and separation by centrifugation.
(~50 kDa) that was slightly higher than the predicted molecular weight (41.5 kDa). Analysis of the soluble fraction of cells expressing mthsp70-ATPase shows a small band at the same molecular weight indicating that the ATPase domain may be partially soluble. However, the band migrating at an apparent molecular weight of ~50 kDa in the soluble fraction is likely the background expression of an endogenous protein in E. coli (see Figure 3.6) and human mthsp70 ATPase domain is likely completely insoluble. This suggests that the human mthsp70 ATPase domain may also be the domain requirement for insolubility and require Hep1 for solubility of the entire protein corroborating the data previously obtained for yeast mthsp70 (Sichting, M. et al. 2005).
CHAPTER 4

HSCB RESULTS

4.1 Human HscB is a mitochondrial protein

Previous studies have demonstrated that *E. coli* HscB is a J-type co-chaperone which targets the substrate protein IscU, the Fe-S cluster scaffold protein, to the hsp70-type chaperone HscA to transfer Fe-S clusters from IscU to apo-acceptor proteins (Chandramouli, K. and M.K. Johnson, 2006). In previous studies, yeast HscB has been shown to be translocated to the mitochondria where it regulates chaperone-assisted Fe/S-cluster biosynthesis (Voisine, C. et al. 2001). To determine if human HscB is a mitochondrial protein, a mammalian expression vector for producing eGFP fused to the C-terminus of HscB was generated by cloning full length HscB (that encoded by all six of its exons; see Sun, G. et al. 2003) into pEGFP-N1. This vector was transiently transfected into HEK 293 cells, a tissue culture line that is mitochondrial rich, and the localization of human HscB was characterized using confocal microscopy (special thanks to Ramsey Kamar). Figure 4.1 shows the results from these studies. The eGFP signal appears to be localized with that of MitoTracker Red CMXRos (Molecular Probes), a dye that stains mitochondria by detecting the membrane potential (Poot, M. et al. 1996). This finding provides evidence that human HscB is localized to the mitochondria. However, it does not exclude a role for HscB in regulating cytosolic Fe/S-cluster biosynthesis.

4.2 Recombinant human HscB is a soluble monomeric and α-helical protein

To obtain large amounts of human HscB for biochemical studies bacterial expression vectors for producing full length HscB with a C-terminal his tag, full length HscB without any tag, and HscB lacking its Cys-rich domain [HscB(71-236)] were
Figure 4.1. Human HscB localizes to the mitochondria. HEK 293 cells transfected with pEGFP-HscB were incubated for 45 minutes in 100 nM MitoTracker Red CMXRos and analyzed live on a Zeiss LSM confocal microscope. Images of HscB-eGFP and MitoTracker Red were collected and colocalization in overlaid image appears as yellow to orange depending on the ratio of the merged fluorescence.
created. The HscB encoded by each of these vectors is soluble when expressed in *E. coli* (data not shown). In addition, human HscB(71-236) was purified and its secondary structure and apparent molecular weight evaluated. The far UV circular dichroism spectra of HscB(71-236) was found to be highly helical showing minima at 208 and 222 nm like *E. coli* HscB (Cupp-Vickery, J.R. and L.E. Vickery, 2000). Figure 4.2 shows the far-UV CD spectrum for purified human HscB(71-236). Using the K2D algorithm, HscB was estimated to contain 84% α-helix and 16% random coil (Andrade, M.A. et al. 1993). The oligomeric state of HscB(71-236) was also investigated. Human HscB(71-236) eluted from a Superdex200 size-exclusion column (see Figure 4.2) at a volume corresponding to a molecular weight of ~28 kDa. This suggests that human HscB(71-236) is monomeric in solution like *E. coli* HscB as it has a calculated molecular weight of ~20 kDa and likely has a slightly elongated conformation (Vickery, L.E. et al. 1997).

4.3 *M. magneticum* HscB contains a chromophore

Human HscB has an N-terminal domain (76 residues) not found in *E. coli* or *S. cerevisiae* HscB homologs, which contains two CxxC motifs commonly found in metal binding proteins. Many multicellular organisms contain such Cys-rich motifs, and four bacteria have HscB homologs with an extra domain having this motif. One such organism is *Magnetospirillum magneticum*, a deep sea magnetotactic bacteria that harbor magnetosomes, specialized organelles containing magnetite (Bazyliński, D.A. and R.B. Frankel, 2004). To determine if the tetracysteine motif is an iron binding site, the binding of the protein to a cofactor was examined. The full-length *M. magneticum* HscB was cloned into pET21d(+), an IPTG inducible expression plasmid that produces HscB with a C-terminal His-tag. *M. magneticum* HscB was found to be soluble upon heterologous
Figure 4.2. Human HscB(71-236) is a highly α-helical and monomeric protein. *Left,* Far-UV CD spectra of 6 μM human HscB(71-236) from 200 to 250 nm. Insert shows the structure of *E. coli* HscB [PDB 1FPO] from (Cupp-Vickery, J.R and Vickery, L.E. 2000). *Right,* Chromatogram from Superdex200 size exclusion chromatography of human HscB(71-236). Inset shows a comparison with molecular weight standards.
overexpression in Rosetta2 *E. coli*. Additionally, purification by nickel talon affinity chromatography revealed that the mmHscB was colored upon purification, suggesting that mmHscB had a chromophore bound. Loading the column with the soluble cell lysate produced a purple colored resin from the blue NTA resin. Elution with imidazole washing buffer yielded a reddish-brown color. This color disappeared upon overnight exposure to the air. Figure 4.3 shows the images of the column before loading, after loading, the eluted sample, and the eluted sample after overnight exposure to air. Additionally, Figure 4.3 shows the UV-VIS spectrum taken immediately following elution.
Figure 4.3. mmHscB has a chromophore bound on purification. Top from left to right, the NTA column before loading, after loading, the eluted sample, and the eluted sample after overnight exposure to air. Bottom, UV-VIS spectra from 225 to 700 nm for the elution buffer (blue) and the eluted protein sample (red).
CHAPTER 5
DISCUSSION AND PERSPECTIVE

5.1 S. cerevisiae Hep1 and human mthsp70

Studies performed in S. cerevisiae have demonstrated Hep1 to be an essential protein and implicate zinc binding as essential for in vivo function. Deletion mutants of Hep1 can be rescued by a single-copy plasmid coding for Hep1 (Burri, L. et al. 2004; Yamamoto, H. et al. 2005; Sichting, M. et al. 2005), whereas the Hep1 cysteine mutants, C75S and C100S, rendered the cells inviable (Yamamoto, H. et al. 2005). In addition, a recent structural study noted a requirement of zinc in the buffer for maintenance of the structure of the core domain (residues 64-159) which was resistant to trypsin digestion (Momose, T. et al. 2007). The results described herein provide additional evidence that Hep1 coordinates zinc with its four conserved cysteine residues. I found that coordination of the zinc cofactor is a requirement for solubility. Heterologous expression of Hep1 demonstrated that mutation of any of the four conserved cysteine residues to serine produced an insoluble Hep1 product. Expression of Hep1 in minimal media containing inadequate or no zinc supplement also affected Hep1 solubility. Expression in medium lacking zinc produces insoluble Hep1 as observed for the cysteine mutants. In vitro elemental analysis of purified Hep1 provided direct evidence that Hep1 contains stoichiometric amounts of zinc. In addition, MMTS/PAR colorimetric detection displayed a 4:1 reacted sulfhydryl to released zinc ratio. Taken together, these findings provide evidence that Hep1 requires four conserved cysteine residues to bind zinc for solubility.
(Momose, T. et al. 2007). This data suggests that Hep1 is specific for mitochondrial hsp70s.

The mechanism by which Hep1 interacts with mthsp70 to promote the production of soluble mthsp70 may involve binding to and stabilizing the folded state, binding to a folding intermediate, or binding to the unfolded state to prevent oligomerization and off-pathway aggregation. The observance of little change in the thermal transition temperatures for mthsp70 melting upon addition of Hep1 indicates that Hep1 does not increase the thermostability of mthsp70. Further experiments examining the effect of Hep1 on mthsp70 folding and function will be required to establish the mechanism of regulation.

Mutants of yeast Ssc1 demonstrated that a mutation in the ATPase domain affected Hep1 interaction but a mutation in the PBD did not. This was corroborated by ATPase domain insolubility in the presence or absence of Hep1 and solubility of the PBD in the absence of Hep1 (Sichting, M. et al. 2005). Taken together this data seems to suggest that Hep1 interacts with the ATPase domain but also requires the PBD. The ATPase domain of human mthsp70 also insoluble when expressed separately, further suggesting a Hep1 and ATPase domain interaction. Continuing studies in our lab using chimeras of E. coli HscA and human mthsp70 established that the ATPase domain is required for the insolubility of mthsp70 and that Hep1 is sufficient to maintain the solubility of a chimera containing the human mthsp70 ATPase domain (Zhai, P. et al. 2008).

5.2 Human HscB and M. magneticum HscB
Human HscB was shown to be mitochondrially localized as predicted based on experiments performed in *S. cerevisiae*. However, differences between the yeast and human HscB may play an important role in the regulation of iron-sulfur cluster biogenesis. Human HscB contains an N-terminal domain of 76 residues in which there is a putative metal binding motif composed of a tetracysteine motif. This motif is not found in *E. coli* or *S. cerevisiae* homologs but is conserved among mammals, some eukaryotes, and a handful of bacteria including *M. magneticum*. The purified mmHscB was found to coordinate a chromophoric iron which was air sensitive. This may indicate that the iron center is designed to detect the oxygen level and repress activity of HscB when there is no oxygen present. Unfortunately, iron has not been detected on HscB lacking a His tag and it remains unclear how our recombinant HscB coordinated iron.

Iron-sulfur clusters can be built spontaneously under anaerobic conditions and many anaerobes do not have the chaperone and co-chaperone components of the iron-sulfur cluster biosynthesis machinery (Ali, V. et al. 2004). The need for additional regulation remains unclear although it may prevent futile ATP hydrolysis at the protein level of regulation when the organism or tissue is experiencing anaerobic conditions. *M. magneticum* are deep sea magnetotaxic bacteria that live under anaerobic and aerobic conditions. Human tissue also operates under anaerobic and aerobic conditions during various levels of physical activity. Perhaps the evolution of the ability to regulate iron-sulfur cluster biosynthesis at the protein level confers an additional advantage to these organisms by conserving ATP. Figure 5.1 shows a cartoon schematic for the proposed regulatory role of the Cys-rich domain of mmHscB in iron-sulfur cluster biosynthesis.

5.3 Perspectives
Figure 5.1. A cartoon schematic for the proposed regulatory role of the Cys-rich domain of mmHscB in iron-sulfur cluster biosynthesis. Clockwise from bottom right, hsp70 ATPase cycle for the HscA with ATP binding leading to a low affinity state for substrate. HscB recruiting the substrate protein IscU with Fe-S cluster bound to the chaperone. ATP hydrolysis converts the complex to the high affinity state for substrate protein and transfers the cluster from IscU to the apo-acceptor protein. Then nucleotide and substrate protein (IscU) exchange restart the cycle. The binding of iron to the N-terminal domain may regulate by inhibiting the interaction with IscU or with the chaperone. Thus ATP hydrolysis does not occur which prevents futile cycling when the ISC machinery is not required under anaerobic conditions to produce iron-sulfur clusters.
These studies provide direct insight into Hep1 cofactor requirements, substrate specificity and thermostability. Hep1 has been characterized as a zinc-binding protein coordinated by four conserved cysteines required for Hep1 solubility. The exact molecular mechanism by which Hep1 interacts with mthsp70 to assist folding remains undetermined though indication of interaction with a folding intermediate or unfolded state has been shown. The requirement of the ATPase domain of mthsp70 for insolubility was also demonstrated. Further \textit{in vitro} characterization of human mthsp70 including substrate interaction and structural studies will be facilitated by the methods outlined herein for generating large levels of soluble recombinant protein. This characterization may include analysis of the cochaperone regulation of the ATPase cycle, solution of a crystal structure, and kinetic analysis with potential drug candidates.

Furthermore, these studies with \textit{M. magneticum} and human HscB proteins provide insight into the possible role of an iron cofactor in providing protein level regulation of chaperone-assisted Fe/S-cluster biogenesis. Additionally, the mitochondrial subcellular localization of human HscB was determined. mmHscB was demonstrated to bind an air-sensitive iron cofactor. The continued investigation of the CxxC motif found at the N-terminus of human HscB and \textit{M. magneticum} HscB may lead to the full characterization of a novel iron binding motif and method of regulation in iron-sulfur cluster biogenesis.
REFERENCES


