WILLIAM RYAN SCHWANER
Characterization of *in vivo* Chaperone Function by the Rescue of Nonfunctional Missense Mutants
(Under the Direction of TIMOTHY HOOVER)

Chaperones play a fundamental role in facilitating the proper folding of intracellular proteins. They rescue misfolded or damaged proteins and allow them the opportunity to refold into an enzymatically active form. While previous studies of chaperone proteins have focused on *in vitro* folding characteristics, this research provides an intracellular examination of chaperone function by analyzing the ability of either GroELS or DnaKJ to rescue misfolded proteins *in vivo*. Libraries of non-functional missense mutants were obtained for the *hisC* and *hisD* genes from *Salmonella typhimurium* and the *lacY*, *lacZ*, and *trpA* genes from *Escherichia coli*. Overexpression vectors containing either the *dnaKJ* or *groELS* genes from *E. coli* were introduced into each missense mutant. Twenty-five percent of the 185 inactive missense mutants tested could be suppressed by the overproduction of either DnaKJ or GroELS. The percentage of overlap between the specific mutant alleles that could be suppressed by both chaperones was significant.

INDEX WORDS: Chaperone, Missense, Mutant, GroELS, DnaKJ, *In vivo*

CHARACTERIZATION OF *IN VIVO* CHAPERONE FUNCTION BY THE RESCUE OF NONFUNCTIONAL MISSENSE MUTANTS

by

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Protein Folding

The study of protein folding began with Anfinsen's work in 1973. He proposed that all of the information needed by a protein to reach its tertiary, active structure was found in the amino acid sequence (1). His experiments were conducted shortly after Sanger had determined the sequence of insulin and thereby proven that a protein's basic covalent structure is its amino acid sequence and that this chain is unique for each protein. Anfinsen took this work a step further and showed that a fully denatured polypeptide chain could, under the right conditions, refold to form a native protein regaining full biological activity. This concept holds true today, although it is now known that other components, known as molecular chaperones, may aid in this process.

A nascent polypeptide faces many obstacles on its path from the ribosome to an active structure. *In vivo* conditions, including temperature and pH, or the presence or absence of various nutrients, growth factors, or inhibitors can have effects on the efficiency of the protein folding process (11). Additionally, the presence or absence of proteases will determine the chances of a protein reaching its native three-dimensional structure (33). While there is much debate (10,18) as to the intricacies of each conformation a polypeptide might take in reaching an active structure, the overall pathway of the reaction is relatively simple. Figure 1 outlines the steps a polypeptide takes from creation to destruction or salvation.

As a polypeptide is formed on the ribosome, it emerges as a chain of amino acids bound by peptide bonds. This chain is known as the primary amino acid sequence.

There is no three-dimensional structure at this stage due to a lack of complex interactions between the amino acids in a given chain, and the protein remains inactive. From this point, the chain can move on to a molten globule structure, which may contain some secondary structure including α-helices or β-pleated sheets (42). This stage is described as molten globule because the chain is in a constant state of flux, moving from one potential structure to another. These structural changes are driven by thermodynamic considerations in which the polypeptide seeks a conformation that is energetically favorable. Finally, the polypeptide will form the tertiary, or active conformation, but only if the cellular conditions required for each protein are met. At any step along this process, problems may occur. For example temperature or pH levels may not be optimal for the folding energies required for the polypeptide chain in question. When such problems are encountered, chaperones can facilitate proper folding of the protein, or proteases can degrade the protein.

If this scenario were a one-way system, much of the cell's valuable energies would ultimately be lost to protease degradation. It may prove beneficial then if proteins could repeatedly fold and refold if problems were encountered. This is not often the case, however, due to the extensive lengths of some polypeptides. Refolding experiments often show the formation of kinetically trapped intermediates that aggregate, even in dilute aqueous solutions or in low temperatures (32). When folding is attempted under physiological conditions, aggregation becomes an even greater problem due to the hydrophobic interactions that take place in a denatured protein. The high concentration of other macromolecules in the cell also creates conditions that promote aggregation (11). In fact, a common method for increasing the available level of active protein *in vitro* is to maintain low protein concentrations during the re-folding process.

In the mid 1980's, several components of the protein folding pathway were discovered. Enzymes catalyzing reactions such as disulfide interchange and proline *cis* to *trans* isomerization were further characterized (17). Other proteins occasionally requiring the hydrolysis of ATP were also discovered, including the chaperone and protease families. It is now believed that the presence of some of these 'helper' enzymes prevents the initial folding of proteins to assist in targeting them to their correct location in the cell.

Heat Shock Response and its Regulation

In 1962 F. Ritossa discovered a novel puffing pattern in the polytene chromosomes of the fruit fly *Drosphila buschii* after a heat shock (49). Today this characteristic is known as the heat shock response, and all organisms have an intricate system of proteins capable of dealing with its detrimental effects. These heat shock proteins (Hsps) are the result of a dramatic change in the pattern of gene expression in the cell and can be triggered by a variety of stress factors. They are ubiquitous, occurring in all manners of organism from bacteria to humans. The most likely signal for induction of this response is the accumulation of misfolded proteins in the cell, although the exact mechanism is still unknown. Many of the Hsps are chaperone proteins and their increased levels in the cell post heat shock gives the cell a means for dealing with a potentially lethal stress condition. Sometimes a stress can be too great, however, and the heat shock response is also responsible for the triggering of the cell death cycle. The relationship between Hsps and the apoptosis pathway are not entirely understood, but it is well-known that both Hsp27 and Hsp70(DnaKJ) play strong antiapoptotic roles (31).

In *E. coli*, a temperature upshift from 30 to 42ºC is all that is needed to produce a rapid, up to 15-fold, induction of synthesis of over 20 Hsps. This is followed by an adaption period where the Hsp synthesis rate levels off at a new, higher steady-state level

(4,16). This response is controlled in *E. coli* at the transcriptional level by the product of the *rpoH* gene, the heat shock promoter specific σ^{32} subunit of RNA polymerase (23). σ^{32} is required for induced expression of the Hsps, as well as for basal levels. The cellular concentration of σ^{32} is very low under steady-state, non-stressed conditions and is limiting for heat shock gene transcription. Upon a stress situation, a rapid increase in σ^{32} levels, and possibly its activity, leads to expression of the Hsps. Both the DnaK and DnaJ chaperone proteins are known to bind with σ^{32}. Under stress conditions, more and more of DnaK and DnaJ are bound to damaged proteins, causing more σ^{32} to be available for up-regulation of the heat shock proteins. Shut off is managed by a lowering of σ^{32} levels and this quick and thorough regulation system allows *E. coli* to rapidly adapt to a changing environment (9).

Chaperones

To understand further the processes involved in protein folding, we must take a detailed look at chaperones. One of the first articles to present any detailed description of chaperones gives the following description of molecular chaperones (11):

'Molecular chaperones are proteins whose role is to mediate the folding of certain other polypeptides and, in some instances, their assembly into oligomeric structures, but which are not components of these final structures.'

What is important to note is the idea of assisted self-assembly. The notion of a protein's folding pathway being dictated by its primary structure, as Anfinsen's work suggested in 1973, is not lost here. Rather, the chaperone concept is concerned with efficiency of operation as opposed to direct folding assembly. Additionally, prevention

of missfolding of proteins is a primary role of the chaperone system. The name is quite fitting, as the role of a human chaperone is to prevent inappropriate interactions between minors. Molecular chaperones provide a stable, energetically more favorable environment in which a protein has a better chance to fold correctly. These proteins are behaving like enzymes, in that they do not cause reactions to take place that would otherwise not occur, but are accelerating the rates at which such reactions take place. While several proteins of unknown function have been described as chaperones, there are two major families of ATP-dependent chaperones. These are the chaperonins (Hsp60 class) and the Hsp70 class of proteins (5).

GroELS

GroELS is a member of the chaperonin, or Hsp60, class of proteins. Originally described as a sequence-related class of molecular chaperones (27), chaperonins have been found in a wide variety of organisms and are distinguished by there ring structure composed of several subunits. These include the bacteria, archae, mitochondria (including those from yeast, *Drosophelia*, and human cells), and plastids (chloroplasts and chromoplasts). The degree of sequence relatedness is quite high among the chaperonins. For example, the yeast mitochondrial chaperonin 60 protein shares 45% identical residues with the wheat plastid chaperonin, and 54% identical residues with *Escherichia coli* Hsp60 (53).

Chaperonins are large, cylindrical protein complexes that consist of two stacked rings that each contain seven to nine subunits (4). These rings, when viewed in their active structure via X-ray crystallography, appear as two doughnuts stacked one on top of the other. Within the chaperonin family, there are two main groups. Group I chaperonins include the GroEL protein from *E.coli* and the mitochondrial Hsp60. These chaperonins

work in concert with a ring-shaped protein, GroES in the case of GroEL, and Hsp10 in the case of Hsp60. This seven-subunit cofactor sits on top of the ringed structure and acts in concert with the binding and release of substrate and protein to the complex.

Proteins belonging to the chaperonin class, like other chaperones, are expressed at very high levels in the cell after a stress response. Increased amounts of chaperonin have been described after heat shock (11), bacterial infection of macrophages (6), and increased cellular levels of unfolded, or aggregated proteins (46). Many studies have been performed trying to estimate the extent to which chaperonins aid in the protein folding process. Results from experiments that examined the portion of cellular proteins that bind to the chaperonins vary. Estimates for the interaction with other proteins for *E. coli* GroEL range from 2-4% of the cellular proteins (41), up to as high as 30% (29). These differences are due to discrepancies between preparation methods of the studies performed. Direct experiments that examined the flux of newly synthesized proteins through GroEL showed that this chaperonin interacts with approximately 12% of *de novo* formed protein. This figure increases 2- to 3-fold when the cells are subjected to a heat shock (13). The majority of the substrates that GroEL interacts with are in the range of 10 and 50 kDa (30). This size restriction is consistent with the constriction of the interior dimensions of the GroEL ring structure itself.

Several models have been proposed for the mechanism of GroELS-mediated protein folding. Ideas range from the progressive binding of more native-like states during repeating rounds of substrate cycling within the GroELS complex (42), to complete unfolding of the substrate polypeptide on the inner chaperone surface (63). One widely accepted model (57) proposes that the GroEL complex is composed of two seven-member rings stacked on top of each other, the *cis* and *trans* rings. The nacent polypeptide binds to several hydrophobic residues located in the apical domains of the *cis*

ring, after which ATP binds to the *trans* ring along with the binding of GroES to the top of the *cis* GroEL ring (see Figure 2). These actions cause a conformational change in the *cis* ring effectively increasing the size within the GroELS chamber. Binding of ATP has also been shown to increase the flexibility of the bound substrate inside the complex (55). Once ATP and GroES are bound to the GroEL complex, the substrate is released and allowed to fold. When ATP hydrolysis occurs in the *trans* ring, GroES is released, allowing the polypeptide to leave the chaperone complex. The polypeptide is now in one of two states; one committed to a proper folding pathway, or one that may require subsequent rounds of GroEL/ES binding and release.

DnaKJ

Another large class of chaperones is the Hsp70-type proteins, the prime example being *E. coli* DnaK. The Hsp70 chaperone system, including the co-chaperones of the DnaJ/GrpE family, is thought to recognize short extended peptide sequences enriched in hydrophobic residues. Such hydrophobic regions are probably present in all unfolded proteins (21). DnaK is believed to help stabilize these newly formed polypeptides and to prevent their aggregation before proper folding can occur. Like the chaperonins, the Hsp70 class chaperones have ATPase activity and are believed to release their bound substrate upon ATP binding and hydrolysis (26). The ATPase activity of *E. coli* DnaK is regulated by the co-chaperones DnaJ and GrpE (40), although most of the *in vitro* work to date on this system has been carried out in the absence of these co-chaperones (47).

Like the chaperonins described earlier, the Hsp70 class proteins are highly conserved. DnaK shares about 50% amino acid identity with the Hsp70 proteins found in eukaryotes (39). All family members have a highly conserved amino-terminal ATPase domain of approximately 45-kDa and a carboxy-terminal which is divided further into a

15-kDa substrate-binding domain and a 10-kDa subdomain of unknown function (25). Some DnaK-type proteins contain short amino- or carboxy-terminal extensions that are required for targeting to specific cellular compartments or membranes. For bacteria, the main Hsp70-type protein is DnaK. A second protein of high homology to DnaKJ has been identified in *E. coli.* This protein, known as HscA, or Hsc66, is thought to perform many of the same functions of DnaK, although it is controlled under a cold-shock response (38). There are at least 14 Hsp70 types in yeast, including the cytosolic proteins SSA1 and SSA2 and the mitochondrial protein Ssc1p. In mammalian cells, there is the cytosolic hsp70 and prp73, as well as the endoplasmic reticulum protein Bip.

Hsp70 family chaperones, while known to aid in the folding of nacent polypeptide chains, aid in several additional cellular functions. These include the import of proteins into cellular compartments and the folding of proteins in the cytosol, endoplasmic reticulum and mitochondria. Additionally, Hsp70 proteins play a large role in controlling the activity of regulatory proteins, including feedback regulation of the σ^{32} system in *E.coli*. σ^{32}, a transcription factor encoded by *rpoH*, is responsible for the regulation of several heat shock proteins including GroEL, DnaK and the Lon protease (62). Finally, DnaK has also been shown to be involved in the dissolution of protein complexes and the degradation of unstable proteins (51).

The current model describing the chaperone function of the DnaKJ system is as follows (see Figure 3). In the absence of a protein substrate, only weak interactions exist between DnaK and DnaJ. Under physiological conditions, DnaK is bound to ATP and has a low affinity for unfolded polypeptides. The first protein in the system to bind unfolded polypeptide chains is DnaJ. The DnaJ/peptide complex activates the ATP-hydrolytic activity of DnaK and stabilizes the ADP-bound state of DnaK. DnaJ is released from the complex at this point, leaving a DnaK-peptide ternary complex. The

co-chaperone GrpE binds to the complex, initiating the interaction of DnaK with the polypeptide. When GrpE binds, it causes DnaK to again bind ATP, which returns DnaK to the low-affinity state, causing the release of the polypeptide from the complex. GrpE, ADP, and the polypeptide are then released from DnaK. At this point the polypeptide may proceed to fold properly, continue on to another chaperone machine, such as GroELS, or it may rebind to DnaJ to start the cycle again (26).

Summary of Research

Chaperones aid in the intracellular folding of proteins. It is no wonder, then, that chaperones have been the focus of much research aimed toward the optimization of active, overproduced proteins. Overproduction of proteins, however, is often a very inefficient process, as mass quantities of any protein in a living system is rarely tolerated, and many protein products often end up degraded or trapped in inclusion bodies. Fortunately, both *in vitro* and *in vivo* studies have shown that the overproduction of certain chaperones can aid in the folding of target proteins. An excellent review on the subject is presented by Thomas *et al.* (53).

In vitro studies of chaperone folding activities often involve simple refolding assays on the target protein. The protein in question is first denatured by physical or chemical means. Purified GroELS or DnaKJ is then added to the system, along with ATP, and folding rates and enzyme activities are measured. *In vivo* studies generally include the overproduction of one or more chaperone systems while looking for increased solubility or secretion of the desired protein product. Table 1 lists several of these studies performed to date. Upon examination of these studies, several problems emerge. First, compared to the number of experiments focusing on the GroELS system, DnaKJ is greatly under-represented. Relatively few studies, either *in vivo* or *in vitro*, have been

done to test the affects of DnaKJ. Second, of the studies that involve DnaKJ, the effect of GrpE on this system is practically ignored, although it is known to play an essential role in the chaperone mechanism. Finally, very few studies have directly compared the ability of GroELS versus DnaKJ to fold or rescue unfolded proteins.

For all of the reasons just listed, I developed a more comprehensive, *in vivo* approach to analyze chaperone function. The goal of this study was to investigate the ability of over-produced chaperones to activate enzymes that are inactive due to the presence of a single amino acid substitution. This substitution, if in a critical folding site in the sequence, can cause a protein to misfold, not unlike the misfolding caused by excessive heat stress. I used libraries of missense mutants in several different proteins to analyze chaperone ability to suppress these mutations by allowing the proper folding to occur. All totaled, I analyzed 185 missense mutants that encoded inactive proteins, of which approximately twenty-percent could be suppressed by overproduction of either GroELS or DnaKJ.

Figure 1. This figure represents the steps polypeptide chains follow on the way to their active structure. Chaperones may act at different stages in this pathway based on their preferences for different binding regions. Proteases may interfere with this process at any stage.

Protein Folding *in vivo*

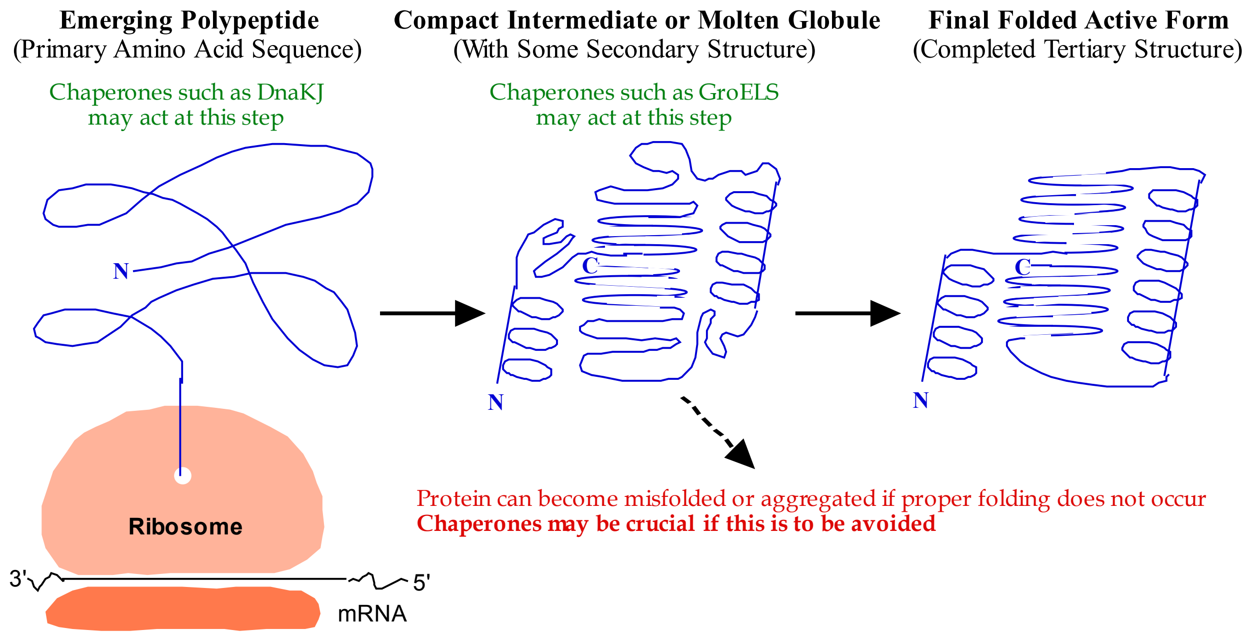


Figure 2. Three-dimensional representation of the GroELS complex. Two GroEL rings sit stacked upon one-another, with a GroES cap bound to one end. The ring with the bound GroES is called the *Cis* ring, opposite ring the *Trans* ring. Each GroEL ring is made up of 7 subunits.

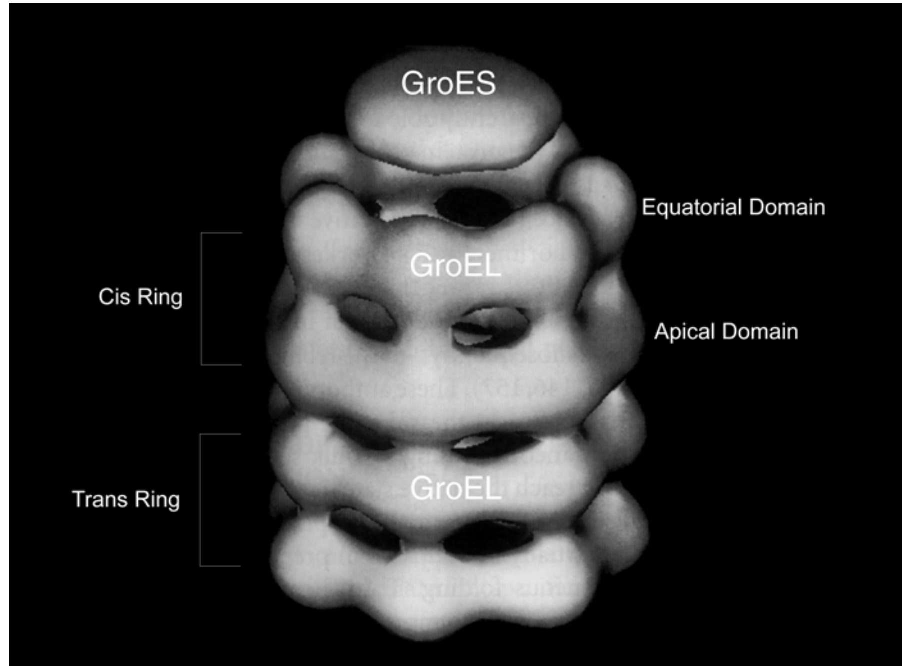

GroES
Equatorial Domain
GroEL
Cis Ring
Apical Domain
Trans Ring
GroEL

Figure 3. Model of GroELS Reaction Cycle. The blue sections represent stacked GroEL structures in cross-section. GroES is represented by the aqua colored cap. ATP and GroES bind to the complex and the chain is now released into a larger central cavity. This cavity forms a protective ‘garage’ in which the chain is allowed to fold on its own. One round of ATP hydrolysis and subsequent binding of ATP to the *trans* ring allows the peptide chain to release into solution, completing the cycle. Multiple reaction cycles may be necessary for a protein to reach its tertiary structure. Adapted from Ellis, 2001 (12).

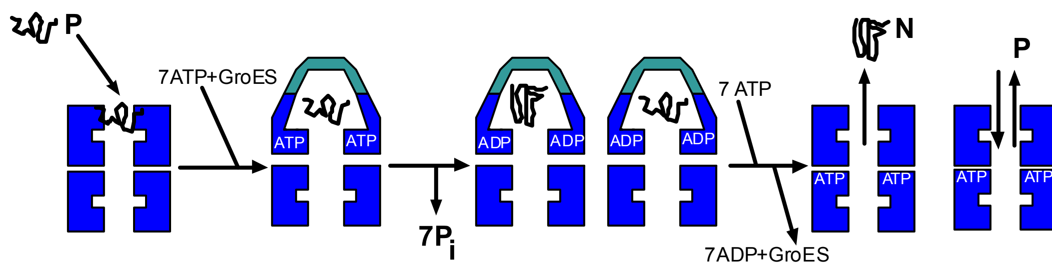

P
7ATP+GroES
ATP
ATP
7P
i
ADP
ADP
ADP
ADP
7 ATP
7ADP+GroES
N
ATP
ATP
P
ATP
ATP

Figure 4. **Model of the Hsp70 reaction cycle**. This model is adapted from the work of Hartl, 1996 (26). DnaJ initiates this process by binding nascent polypeptide chains. This DnaJ/polypeptide complex activates the ATP-hydrolytic activity of DnaK and stabilizes the ADP-bound state of DnaK. GrpE binds to this structure, initiating the interaction of DnaK with the polypeptide. GrpE, ADP and polypeptide are all released. The polypeptide may now proceed to fold properly, continue on to another chaperone machine, or it may rebind to DnaJ and repeat this cycle.

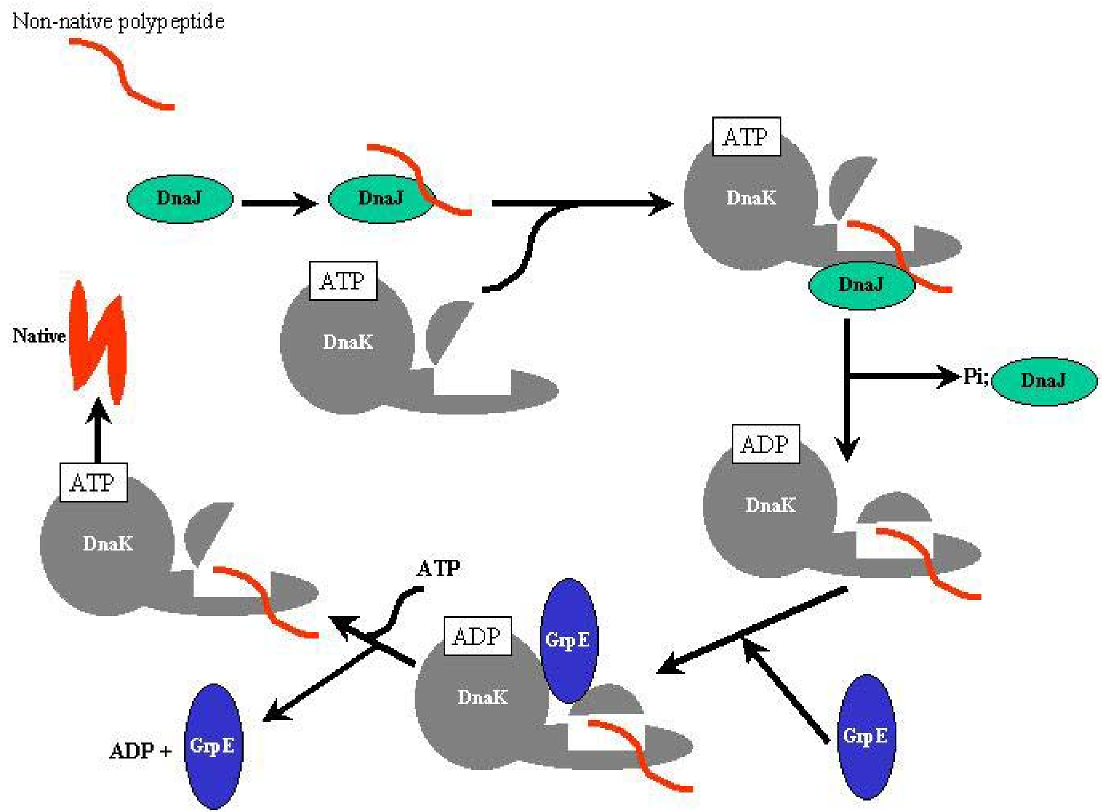

Non-native polypeptide
DnaJ
DnaJ
ATP
DnaK
ATP
DnaK
DnaJ
Pi;
DnaJ
Native
ADP
DnaK
ATP
DnaK
ATP
ADP
GrpE
DnaK
GrpE
ADP +
GrpE

Table 1. Major cytosolic chaperone families of *E. coli*.

Chaperone Family	Structure	ATP	*E. coli* Member	Number of Active Species/Cell	Null Mutant Phenotype	Function
HSP100	6-mer	+	ClpA		No phenotype	Protein degradation together with the ClpP Protease
			ClpB	500	Impaired thermo-tolerance	Disaggregation of protein aggregates together with the DnaK system
HSP90	Dimer	+	HtpG	1050	Reduced growth at 44ºC	Unknown
HSP70	Monomer	+	DnaK co-chaperone DnaJ/GrpE	9900	Temperature-sensitive growth (39ºC)	de novo protein folding Prevention of protein aggregation at high temp. Regulation of heat shock response Disaggregation of protein aggregates together with ClpB
HSP60	14-mer	+	GroEL co-chaperone GroES	1230	Lethal	de novo protein folding Prevention of protein aggregation at high temp.
sHSP	8-24-mer		IbpA IbpB	<600	No phenotype	Prevention of protein aggregation at high temp.
Trigger Factor	Monomer		Trigger-factor	20000	No phenotype	Ribosome associated chaperone de novo protein folding

Adapted from Schlieker *et al*.(50).

Table 2. *In vitro* studies of chaperone function on refolding.

Protein Tested	GroEL	GroES	DnaKJ	Reference
β-galactosidase	+	**NT**	**NT**	(3)
human carbonic anhydrase II	+	+	**NT**	(48)
human pro-urokinase	+	**NT**	**NT**	(61)
firefly luciferase	**NT**	**NT**	+	(39)
catalase	+	**NT**	**NT**	(28)
glycerol dehydrogenase	+	**NT**	**NT**	(36)
mitochondrial rhodanase	+	**NT**	**NT**	(43)
ornithine transcarbamylase	+	+	**NT**	(64)
glucose-6-phosphate dehydrogenase	+	**NT**	**NT**	(24)
glutamine synthetase	+	+	**NT**	(18)
lambda repressor	**NT**	**NT**	+	(2)
tryptophanase	+	-	**NT**	(44)

NT – Not Tested

Table 3. *In vivo* studies of chaperone function on refolding.

Protein Tested	GroEL	GroES	DnaKJ	Reference
S1 dihydrofolate reductase	+	+	**NT**	(10)
tyrosine kinase	+	+	+	(7)
ribulose-bisphosphate carboxylase	**NT**	**NT**	+	(8)
human growth hormone	-	-	+	(5)
E. coli glutamate racemase	+	+	**NT**	(2)

NT – Not Tested

CHAPTER 2

MATERIALS AND METHODS

Strains and Plasmids

The bacterial strains and plasmids that were used in this study are listed in Table 4. Plasmids were purified using Qiaprep miniprep kits from Qiagen (Qiagen, Inc. Valencia,CA).

Chemicals, Media, and Reagents

Minimal M9 and Rich Luria-Bertani (LB) media used in this study was prepared as described by Miller, 1972. Both *E. coli* and *S. typhimurium* were grown in LB medium shaken at 37° C. Plasmids pAKL1 and pOF39 both contain a gene encoding for ampicillin resistance. Ampicillin was used in rich media at a final concentration of 100 μg/ml and in minimal media at 50 μg/ml when appropriate to select for bacteria containing these plasmids. Glucose was added to media at a final concentration of 0.2%.

Isopropylthio-β-D-galactoside (IPTG) is an inducer of β-galactosidase production in bacteria. IPTG is used together with the chromogenic substrate 5-bromo-4-chloro-3-indolyl-beta-D-galactoside (X-gal) to determine *lac* gene expression in recombinant methods. IPTG is a chemical analog of galactose which cannot be cleaved by the enzyme β-galactosidase. Thus, it can serve as an inducer for activity of the *E. coli lac* operon by binding and inactivating the Lac repressor. A stock solution, 100 mM, was prepared by

dissolving IPTG in water with subsequent sterile filtration of the solution. IPTG was added to medium after autoclaving to a final concentration of 0.2 mM.

X-gal was added to plates for the LacZ suppression studies. β-galactosidase hydrolyses X-gal forming an indigo-type blue-green precipitate. Thus, bacteria which are positive for this enzyme produce blue colonies when grown in the presence of X-gal. X-gal was added to media at a final concentration of 40 μg/ml. Both X-gal and IPTG were purchased from Diagnostic Chemicals Limited (Diagnostic Chemicals Limited, Oxford, Connecticut).

Transformation and SDS-PAGE

Electroporation techniques were used for transformation with selection on LB plates supplemented with ampicillin. Competent cells were constructed via standard methods. SDS polyacrylamide gel electrophoresis (SDS-PAGE) gels used to verify overproduction of GroELS or DnaKJ were ran as described by Laemmli (37) using Gel apparatus provided by Bio-Rad (Bio-Rad Laboratories, Hercules, CA).

Isolating *lacY* and *lacZ* missense mutants for the suppression study.

Collections of well-characterized non-functional missense mutants are available for the *trpA* gene from *E. coli* and the *hisC* and *hisD* genes from *S. typhimurium*. Several of these mutant proteins were examined to determine if their activities could be recovered *in vivo* by overexpression of chaperones. In addition, *lacY* mutants in *S. typhimurium* and *lacZ* mutants in *E. coli* were isolated to expand our study on recovery of enzymatic activity by overexpression of chaperones. To isolate the *lacY* mutants, an LT2 derivative with a *mudF* transposon insertion containing the wild-type *lac* operon was used. *S. typhimurium* is naturally Lac$^-$, so the only copy of *lac* present in these cells was that on

the *mudF* transposon. Cells were mutagenized with ethylmethanesulfonate (EMS) as per Miller and plated onto tetrazolium medium with lactose as the sole carbon source. One-hundred red Lac^- colonies were isolated and subsequently screened on LB agar that contained X-gal to identify the blue $LacZ^+LacY^-$colonies. The $LacY^-$ mutants were then screened for missense mutants using the methodology employed previously in the characterizations of the *hisC* and *hisD* mutants by Greeb and Whitfield (19,46). Briefly, nonsense mutants were eliminated from the collection by screening with suppressor mutations, while frameshift mutants were eliminated by testing for the ability of ICR to revert the mutants. Thirty-three potential non-functional *lacY* missense mutants were isolated in this screen. To isolate potential *lacZ* mutants the *E. coli* strain CSH27 was used. Cells were mutagenized with EMS and plated onto LB agar that contained X-gal and IPTG. Fifty-seven white Lac^- colonies were isolated. To rule out *crp* or *cya* mutants, the potential *lacZ* mutants were tested for the ability to grow on M9 minimal salts media with glycerol as the sole carbon source. Amber and ochre mutants were then eliminated from the collection by screening with suppressor strains. Twenty-nine putative *lacZ* missense mutants were isolated in this screen. Table 5 lists the type and number of missense mutants used in this study.

Suppression of non-functional *hisC*, *hisD*, *trpA*, *lacY* and *lacZ* missense mutants by overproduced DnaKJ or GroELS.

GroELS and DnaKJ were individually overproduced in mutant cells by introducing the plasmids pOF39 and pAKL1, respectively. GroELS is overproduced constitutively from pOF39 while DnaKJ is under control of the *tac* promoter in pAKL1 (47,13). Maximal levels of DnaKJ were obtained by the addition of IPTG at a final concentration of 0.2 mM to the media. Figure 5 shows an example of the levels of

overproduction we obtained by these methods. To move these two plasmids into *E. coli* recipient cells, plasmid DNA from ALS225 was transformed into cells made competent via treatment with calcium chloride. To move these two plasmids into *S. typhimurium* recipients, the plasmid DNA from ALS225 was first moved into ALS5994 in order to bypass the restriction/modification barriers between strains. This plasmid DNA was used to transform electrocompetent cells that had been prepared from the mutant strains.

Plasmids pOF39 and pAKL1 were introduced into the *E. coli* and *S. typhimurium* mutant strain listed in Table 4, and the ability of overproduced GroELS or DnaKJ to restore enzymatic function was examined. In the case of *hisC*, *hisD* and *trpA*, mutant cells in which GroELS or DnaKJ were overproduced were patched onto M9 minimal salts medium with glycerol as the sole carbon source. The plates were then incubated at 37°C and suppression was scored at 1,2,4,7, and 14 days. The suppression studies on *lacY* alleles were carried out as described above except that M9 minimal medium with lactose as the sole carbon source was used. Suppression of the *lacZ* mutants was carried out as described, except that LB agar supplemented with IPTG and X-gal was used. Figure 6 shows an example of a suppression experiment where GroELS was overproduced in a *his*D missense mutant. In this example, the mutant *hisD*450 grew after overexpression of GroELS, showing increased chaperone levels may help rescue missfolded proteins.

Table 4. Bacteria, Plasmids, and Phage Used in this Study

Strain or Plasmid	**Description**	**Source or Reference**
Bacteria		
ALS224	MC1061 F-araD139 DEL(araABOIC-leu)7679 DEL(lac)x74 galU galK rpsL hsr- hsm+	Altman Lab
ALS225	MC1061 FlacIqZ+Y+A+	Altman Lab
ALS240	MC1061 $pOF39	Altman Lab
ALS241	MC1061 $pAKL1	Altman Lab
Plasmids		
pAKL1	*dna*KJ+	Altman Lab
pOF29	*gro*ELS+	Altman Lab
pTHK201	*hsc*AB+	Thomas Kawula (35)
pUC9	Standard overexpression vector, amp^R	Altman Lab
pHscAB	*hsc*A and *hsc*B cloned into pUC9	Altman Lab
Phage		
HT105	P22 transducing phage in *S. typhimurium*	Altman Lab

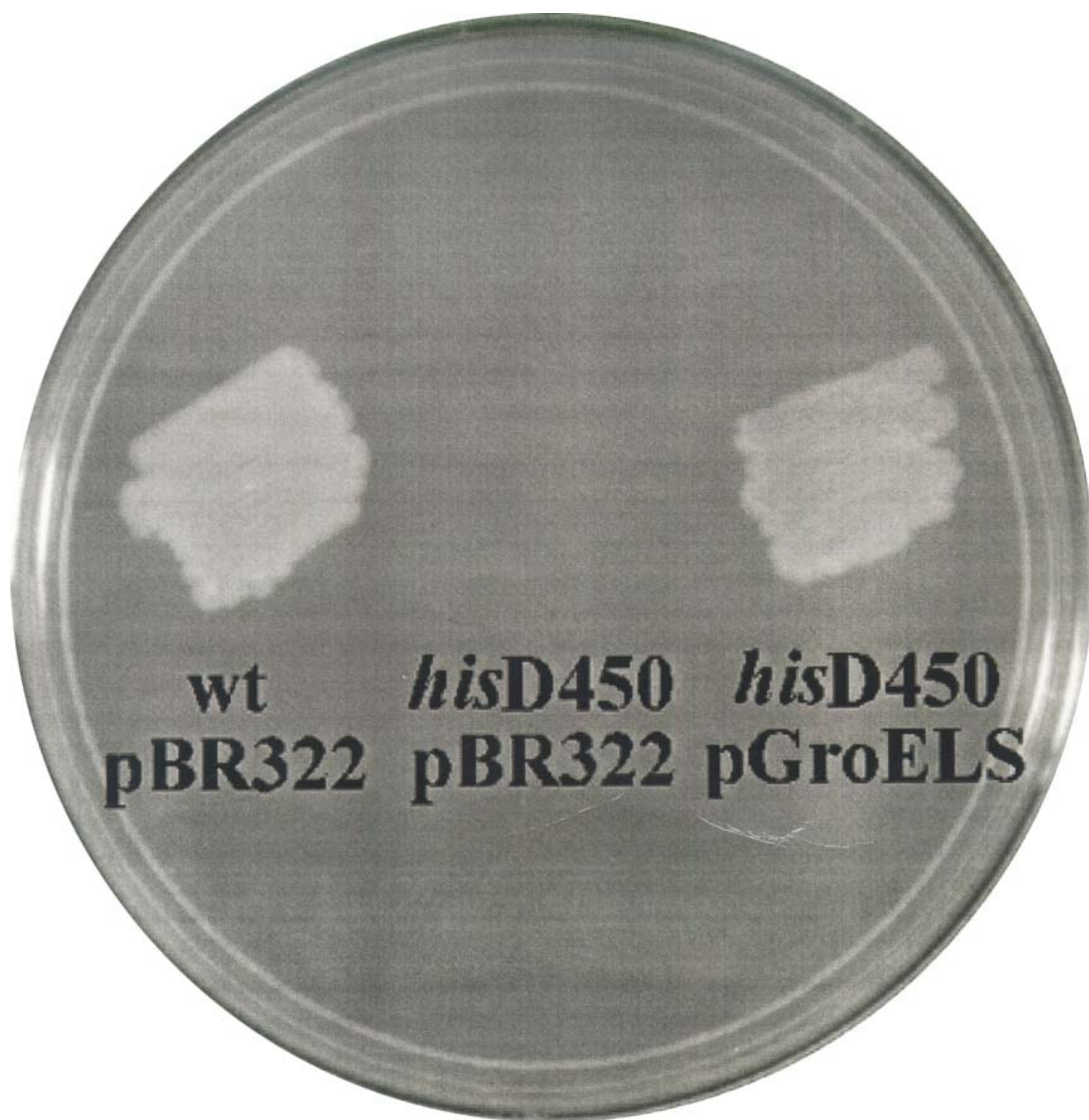
Table 5. Nonfunctional Missense Mutants Used in this Study

Gene	Bacterial Source	Number of Mutants Tested	Laboratory from which Mutants Obtained	Reference
*his*C	*S. typhimurium*	12	Roth Laboratory	(58)
*his*D	*S. typhimurium*	57	Roth Laboratory	(22)
*lac*Y	*E. coli*	70	Altman Laboratory	this study
*lac*Z	*E. coli*	29	Altman Laboratory	this study
*trp*A	*E. coli*	7	Murgola Laboratory	(45)

Figure 5. Overexpression of DnaK as produced by the pAKLI plasmid. Lane one contains our host strain ALS225 grown to the mid log growth phase at 37°C in rich media. Lane two is ALS225 with pAKL1. Lane three is ALS225 with pAKL1 grown in the presence of 1.0mM IPTG to induce expression of DnaKJ. The levels of protein shown here are representative of the levels used for both GroELS and DnaKJ in these experiments.

1
2
3
kDa
116
97
66
58
45
29.2
25.7
DnaK

Figure 6. A plate demonstrating the suppression of *hisD*450 by the overproduction of GroELS. Strains patched onto M9 minimal glycerol media (*his*$^-$). PBR322 was used as a control plasmid carrying ampicillin resistance. WT represents our wild type strain ALS225. Suppression occurring within two days was scored as a "++", and "+" after two days. In this example, the *hisD*450 mutant would not grow unless it was transformed with a plasmid producing excess levels of the GroELS chaperone complex.


wt
pBR322
hisD450
pBR322
hisD450
pGroELS

CHAPTER 3

RESULTS AND DISCUSSION

In recent years, overexpression of chaperone proteins as a means to increase stable protein production has been proposed for both academic and industrial pursuits. One of the key questions that remain unanswered is that of chaperone selectivity. Do all chaperones bind to all proteins or are there specific chaperone-target protein pairs? Tables 2 and 3 show representative samples of the *in vitro* and *in vivo* studies performed to date. What becomes immediately apparent is the lack of comparative studies that address the question of which chaperones work better. Moreover, most of the studies on chaperone function were conducted *in vitro* and focus on individual components of a chaperone system (i.e. just GroEL or GroES, not both together). My study addressed this second, larger question.

In this study a variety of amino acid substitutions in five different proteins were examined for their ability to be rescued by chaperone overproduction. For some of the alleles, such as the *hisD* mutants, rescue of activity was selected by the ability of chaperone overproduction to allow growth on minimal medium. For other alleles, such as the *lacZ* mutants, rescue was monitored by screening for enzyme activity on a selective medium. Tables 6 through 11 list the suppression results that were obtained for each study. I have demonstrated the direct suppression of otherwise inactive mutants by increasing cellular levels of chaperone proteins.

Approximately 20% of the 185 inactive missense mutants tested could be suppressed by the overproduction of either DnaKJ or GroELS (Table 12). Eighty-five

percent of the mutants suppressed by DnaKJ could be suppressed by GroELS overproduction. Likewise, increased levels of DnaKJ suppressed 72% of the missense mutants that were suppressed by the overproduction of GroELS. Hence, the percentage of overlap between the specific mutant alleles that could be suppressed by both chaperones was significant. This finding alone is significant as it indicates that there are several substitutions at key positions in the proteins that overproduction of a chaperone can fix. These substitutions may affect critical folding regions of the proteins in question, and overproduction of either of the chaperones in this study proved effective at those sites.

Based on the above observations, GroELS and DnaKJ were evenly matched in their ability to suppress these mutants. If we look closer at Table 6, however, and compare the number of "strong" suppressors versus "weak" ones, GroELS appears to be a better chaperone for suppression of *hisC* an *hisD* alleles. Moreover, while many alleles were suppressed by both chaperone systems after two days, the majority of those suppressed by GroELS overexpression grew faster on minimal medium than those suppressed by DnaKJ overexpression.

These studies imply that there are specific types of folding problems that different chaperones are able to effectively address. This is evident in the fact that some missense mutants could be rescued by one chaperone system but no the other, as can be seen by the inability of GroELS to aid in the refolding of LacZ (Table 7). This occurrence could be due to several factors. For rescue of an inactive mutant via chaperone overexpression, the mutation would most likely occur within a site critical to the folding process. Chaperones have been described to function as "safe-houses" in which proteins are given the proper time and conditions (e.g. hydrophobicity) to fold properly (11). Conversely, proteins that do not interact normally with either DnaKJ or GroELS would not be capable of being

rescued. Our studies show an example of this in the case of the LacZ mutants and the lack of suppression with GroELS. Based on the size of LacZ and the diameter of the interior cavity of GroEL where the folding process takes place, it is possible that LacZ is too large to fit inside the GroEL structure (34 ,54).

While we observed a fairly high success rate in suppression of mutations with these two chaperone systems, there were many alleles that remained unaffected. This could be due to several factors. Despite the fact that the chaperones were overexpressed to fairly high concentrations, there still may not be sufficient amounts of chaperone present to re-activate enough protein to give us a suppressed phenotype in our screening system. Alternatively, the specific mutation may be in the active site of the protein, and even if the protein were properly folded, we would never see normal activity. Furthermore, the mutation may have caused a fold or interaction in the secondary or tertiary structure that a chaperone simply cannot correct. Finally, chaperones are known to be involved in recognition and destruction of misfolded proteins and may function in concert with proteases (33). These non-rescuable mutants may have been funneled toward the protease-destruction pathway.

This study has focused directly on the abilities of two well characterized chaperones to aid in the *in vivo* rescue of missense mutations. While there is much yet to learn about the direct effects of overproduction of chaperone proteins on these systems, there is much potential for further discovery using such a system. An obvious next step would be to analyze the effect of chaperone overproduction on proteins where the specific missense mutation sites and three-dimensional protein structure were known. This type of analysis could generate very specific information on the abilities and limitations of different chaperone systems. If a particular allele was known to be a key

folding region in the protein, it could be directly examined via overproduction of a specific chaperone.

Finally, this system could be useful as a method to verify the folding abilities of potential chaperone proteins. As new proteins are discovered, they are often characterized as chaperones based on homology alone, and *in vitro* studies do not often reveal the whole picture. This system could be adopted to determine if a novel protein can assist in the refolding of mutant enzymes and the information gathered from such studies could be compared with that collected from other well characterized chaperones.

Table 6. Suppression of *his*C and *his*D missense mutants by overexpression of GroELS or DnaKJ.

Mutant	**Suppression w/ GroELS**	**Suppression w/ DnaKJ**
*hisC*630	++	+
*hisD*64	+	++
*hisD*68	++	-
*hisD*74	++	-
*hisD*88	++	-
*hisD*111	++	-
*hisD*223	+	+
*hisD*226	++	-
*hisD*237	+	-
*hisD*248	+	+
*hisD*274	++	-
*hisD*295	++	-
*hisD*412	++	-
*hisD*450	++	-

Phenotypic suppression upon overexpression of either GroEL or DnaKJ is indicated as '++' for growth by 2 days, or '+' for growth after 2 days. Lack of suppression is indicated by '-'.

Table 7. Suppression of *lac*Y, and *lac*Z missense mutants by overexpression of GroELS or DnaKJ.

Mutant	Suppression w/ GroELS	Suppression w/ DnaKJ
*lacY*16	+	+
*lacY*49	++	++
*lacY*53	++	+
*lacY*62	+	+
*lacY*74	+	+
*lacY*83	+	+
*lacY*85	+	+
*lacY*88	+	+
*lacY*91	+	+
*lacY*92	+	+
*lacY*94	+	+
*lacY*95	++	+
*lacY*100	++	+
*lacY*105	+	+
*lacY*108	+	+
*lacY*111	+	+
*lacY*113	+	+
*lacY*116	+	++
*lacY*120	++	+
*lacY*121	++	++
*lacY*123	++	+
*lacY*152	+	+
*lacY*157	+	+
*lacZ*173	-	+
*lacZ*174	-	+
*lacZ*190	-	++
*lacZ*211	-	++

Phenotypic suppression upon overexpression of either GroEL or DnaKJ is indicated as '++' for growth by 2 days, or '+' for growth after 2 days. Lack of suppression is indicated by '-'.

Table 8. Suppression of *trp*A missense mutants by overexpression of GroELS or DnaKJ.

Mutant	**Suppression w/ GroELS**	**Suppression w/ DnaKJ**
trpA(AGA211)	++	-
trpA(UGG211)	+	++
trpA(UGU234)	-	++

Phenotypic suppression upon overexpression of either GroEL or DnaKJ is indicated as '++' for growth by 2 days, or '+' for growth after 2 days. Lack of suppression is indicated by '-'.

Table 9. *his*C and *his*D missense mutants not suppressible by GroELS or DnaKJ

Mutant	Suppression w/ GroELS	Suppression w/ DnaKJ
*hisC*8	-	-
*hisC*115	-	-
*hisC*120	-	-
*hisC*163	-	-
*hisC*201	-	-
*hisC*210	-	-
*hisC*367	-	-
*hisC*483	-	-
*hisC*496	-	-
*hisC*537	-	-
*hisC*906	-	-
*hisD*1	-	-
*hisD*10	-	-
*hisD*36	-	-
*hisD*37	-	-
*hisD*39	-	-
*hisD*66	-	-
*hisD*77	-	-
*hisD*78	-	-
*hisD*83	-	-
*hisD*84	-	-
*hisD*90	-	-
*hisD*113	-	-
*hisD*126	-	-
*hisD*150	-	-
*hisD*166	-	-
*hisD*170	-	-
*hisD*214	-	-
*hisD*216	-	-
*hisD*220	-	-
*hisD*226	-	-
*hisD*233	-	-
*hisD*239	-	-
*hisD*244	-	-
*hisD*245	-	-
*hisD*254	-	-
*hisD*271	-	-
*hisD*302	-	-
*hisD*362	-	-
*hisD*363	-	-
*hisD*381	-	-
*hisD*392	-	-
*hisD*451	-	-

Table 9. (Cont.) *his*C and *his*D missense mutants not suppressible by GroELS or DnaKJ

Mutant	**Suppression w/ GroELS**	**Suppression w/ DnaKJ**
*hisD*465	-	-
*hisD*472	-	-
*hisD*490	-	-
*hisD*492	-	-
*hisD*506	-	-
*hisD*601	-	-
*hisD*697	-	-
*hisD*698	-	-
*hisD*710	-	-
*hisD*986	-	-

Phenotypic suppression upon overexpression of either GroEL or DnaKJ is indicated as '++' for growth by 2 days, or '+' for growth after 2 days. Lack of suppression is indicated by '-'.

Table 10. *lac*Y and *lac*Z missense mutants not suppressible by GroELS or DnaKJ

Mutant	Suppression w/ GroELS	Suppression w/ DnaKJ
*lacY*1	-	-
*lacY*3	-	-
*lacY*6	-	-
*lacY*14	-	-
*lacY*15	-	-
*lacY*19	-	-
*lacY*24	-	-
*lacY*26	-	-
*lacY*28	-	-
*lacY*29	-	-
*lacY*31	-	-
*lacY*35	-	-
*lacY*36	-	-
*lacY*38	-	-
*lacY*41	-	-
*lacY*51	-	-
*lacY*55	-	-
*lacY*57	-	-
*lacY*58	-	-
*lacY*60	-	-
*lacY*64	-	-
*lacY*68	-	-
*lacY*71	-	-
*lacY*115	-	-
*lacY*142	-	-
*lacY*144	-	-
*lacY*153	-	-
*lacY*154	-	-
*lacY*166	-	-
*lacY*169	-	-
*lacY*170	-	-
lacY173	-	-
lacY175	-	-
lacY176	-	-
lacY177	-	-
lacY178	-	-
lacY180	-	-
lacY191	-	-
lacY197	-	-
lacY199	-	-
lacY201	-	-
lacY203	-	-

Table 10. (Cont.) *lac*Y and *lac*Z missense mutants not suppressible by GroELS or DnaKJ

Mutant	**Suppression w/ GroELS**	**Suppression w/ DnaKJ**
lacY204	-	-
lacY208	-	-
lacZ178	-	-
lacZ179	-	-
lacZ182	-	-
lacZ184	-	-
lacZ187	-	-
lacZ189	-	-
lacZ191	-	-
lacZ192	-	-
lacZ194	-	-
lacZ195	-	-
lacZ198	-	-
lacZ203	-	-
lacZ204	-	-
lacZ206	-	-
lacZ208	-	-
lacZ210	-	-
lacZ214	-	-
lacZ217	-	-
lacZ219	-	-
lacZ221	-	-
lacZ222	-	-

Phenotypic suppression upon overexpression of either GroEL or DnaKJ is indicated as '++' for growth by 2 days, or '+' for growth after 2 days. Lack of suppression is indicated by '-'.

Table 11. *trp*A missense mutants not suppressible by GroELS or DnaKJ

Mutant	**Suppression w/ GroELS**	**Suppression w/ DnaKJ**
*trp*A(GAG211)	-	-
*trp*A(GAA211)	-	-
*trp*A(AAG211)	-	-
*trp*A(CGA211)	-	-

Phenotypic suppression upon overexpression of either GroEL or DnaKJ is indicated as '++' for growth by 2 days, or '+' for growth after 2 days. Lack of suppression is indicated by '-'.

Table 12. Compiled data from suppression studies with GroELS and DnaKJ for five different genes

Gene	% of missense mutants suppressed by GroELS		% of missense mutants suppressed by DnaKJ		% of missense mutants suppressed by GroELS that are also suppressed by DnaKJ		% of missense mutants suppressed by DnaKJ that are also suppressed by GroELS	
*his*C	1/12	8%	1/12	8%	1/1	100%	1/1	100%
*his*D	13/57	23%	3/57	5%	3/13	23%	3/3	100%
*lac*Y	23/70	33%	23/70	33%	23/23	100%	23/23	100%
*lac*Z	0/29	0%	4/29	14%	N.A.	N.A.	0/4	0%
*trp*A	2/7	29%	2/7	29%	1/2	50%	1/2	50%
Total	39/175	22%	33/175	19%	28/39	72%	28/33	85%

CHAPTER 4

ADDENDUM

As described above, the first step of my suppression studies was to evaluate how the well known chaperones GroELS and DnaKJ acted in this screening system. While GroELS and DnaKJ are the best studied chaperone complexes to date, there exist many other proteins with proven or potential chaperone activity. HscAB is one such protein complex.

HscA was originally described through the isolation of mutations that compensated for the effect of a *hns-1* mutation on *fimA* promoter inversion rates (35). The *hsc*A gene encodes a 66-kDa protein product that is a member of the Hsp70 class of proteins (52). HscA shares sixty-two percent similarity and forty-two percent identity with DnaK. The *hscA* gene is part of a bicistronic operon that is proceeded by the gene *hsc*B encoding a 22-kDa protein. Approximately one hundred bases upstream of the *hsc*B gene start site is a region with weak homology to the σ^{70} -10 and -35 consensus sequences, although the regulatory mechanism of this operon is still unknown. Although it is much smaller than the DnaJ protein and their C-terminal binding regions differ significantly, HscB contains a region of high homology to DnaJ on its N-terminal end. It is believed that HscA and HscB combine to form a chaperone complex like that of DnaKJ.

Further characterizations of HscAB were carried out by Kawula and Levivelt (38). Because the *hns-1* gene (whose activities led to the discovery of HscAB) is induced under cold shock, the temperature response of HscAB was tested. Initial studies via RNA probes to *hsc*A mRNA and RNAse protection assays found no induction upon a shift

from 30°C to 42°C, indicating a lack of heat-shock response. However, when the same studies were conducted with a shift from 37°C to 10°C an eleven-fold increase in mRNA levels was found after three hours time (38). *hsc*A levels were also induced after the addition of chloramphenicol, a response indicative of cold-shock. In addition, the levels of Hsc66 protein were tested via Western blots and cold-shock caused increased protein levels. Finally, it was found that mutations in *hsc*A did not affect the growth rate of *E. coli*, but did not have a negative effect on the overall protein profile, indicating a potential chaperone role.

In 1997, Vickery *et al.* made further characterizations of the HscAB complex, including studying its ATPase activity and additional studies involving heat and cold-shock(56). They described similarities between Hsc66 and Hsp70 proteins in the N-terminal ATPase and C-terminal peptide recognition sites, although the specific peptide binding regions differed, as did the regulatory mechanisms. They found that anti-Hsc66 did not recognize DnaK, indicating that the major epitopes of the two proteins varied. When heat and cold-shock studies were performed, no appreciable effect was noticed in the concentration of HscA, while HscB increased slightly under cold-shock conditions. Hsc66 was found to comprise approximately one percent of total cell protein at 37°C. ATPase activities were measured in comparison to those of DnaKJ, and the activities were higher over a broader range and at lower temperatures for HscA than those for DnaK. Based on the above findings, it was proposed that the HscAB complex functioned as a chaperone during normal "housekeeping" functions rather than under stress responses as in the case of DnaKJ.

To date relatively little research has been done on the HscAB protein complex. While the work done is a good start, none of the experiments directly addressed the question of HscAB's chaperone activity. This role has been suggested, but not proven, as

homology to DnaKJ and the ATPase activities alone are not concrete evidence. If HscAB truly were a chaperone, much less one that plays a significant role under normal cellular conditions, how would it compare to DnaKJ in our *in vivo* suppression studies?

In order to test this question, I first needed to construct an over-expression plasmid for HscAB. I obtained pTHK201 from Thomas Kawula which contained the *hsc*AB genes (35). This vector did not produce HscAB in sufficient quantities for the suppression studies, so I cloned the appropriate regions into pUC9, a standard over-expression vector used in our laboratory. Now *hsc*AB was under *lac* promoter control and could be induced with IPTG. Overproduction of the HscA and HscB proteins was verified via SDS polyacrylamide gels. This vector, designated pHSCAB was then transformed into each of the twenty-nine *lac*Z missense mutants used in the original screen. Suppression studies were performed following the same procedures described earlier and the results are shown in Tables 13 and 14. I took the analysis one step further than before, and tested the β-galactosidase activities of these constructs in comparison to their DnaKJ supplemented counterparts. Overexpression of HscAB suppressed several of the *lacZ* alleles and at least for the *lacZ*211 allele, HscAB was more effective than DnaKJ in its ability to suppress the missense mutation.

While these results are preliminary, they provide an important first step in validating the effectiveness of this screening system for identifying chaperone function. This study is the first step, the proof of concept, and hopefully future work will allow a better understanding of the mechanism of chaperone proteins.

Figure 7. Overexperession of HscA and HscB via cloning into the pUC9 vector. Lane one contains our host strain ALS225 grown to the mid log growth phase at 37°C in rich media. Lane two contains ALS225 with pUC9-hscAB and is grown in the presence of 1.0 mM IPTG for induction. Original genes were obtained from the pTHK201 plasmid from Thomas Kawula.

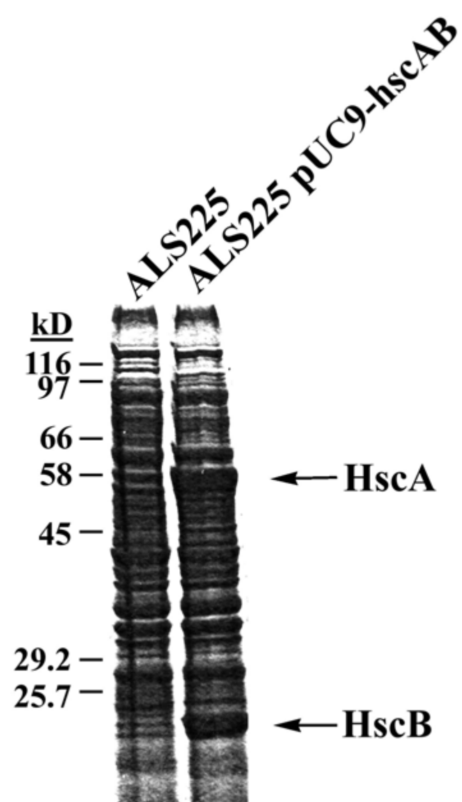

ALS225
ALS225 pUC9-hscAB
kD
116
97
66
58
45
29.2
25.7
HscA
HscB

Table 13. Suppression of lacZ Missence Mutants by DnaKJ and HscAB (Preliminary Data)

Mutant	**Suppression w/ DnaKJ**	**Suppression w/ HscAB**
*lac*Z172	-	+
*lac*Z173	+	++
*lac*Z174	+	++
*lac*Z190	++	++
*lac*Z202	-	+
*lac*Z211	++	++
*lac*Z220	-	+
*lac*Z225	-	++

Phenotypic suppression upon overexpression of either DnaKJ or HscAB is indicated as '++' for growth by 2 days, or '+' for growth after 2 days. Lack of suppression is indicated by '-'.

Table 14. β-galactosidase Activities of *lac*Z Mutants Suppressed by DnaKJ and HscAB (Preliminary Data)

Mutant	**Mutant Alone**	**Mutant w/ DnaKJ**	**Mutant w/ HscAB**
*lac*Z190	**16.05**	**36.73**	**36.92**
*lac*Z211	**3.56**	**51.13**	**643.00**

Numbers given are in standard Miller units. Samples (except mutant alone) were induced with 0.2mM IPTG and grown to mid-log phase.

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