Degradation of the *E. coli* Small Heat-Shock Proteins by the AAA+ Protease Lon: Significance to Protein Quality-Control

by

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ABSTRACT

The refolding and elimination of damaged and aggregated proteins requires the concerted effort of several branches of the protein quality-control network. This network includes refolding chaperones, disaggregases, holdases and proteases. Many years of investigation have led to a partial understanding of how different branches of the protein quality-control network cooperate with each other to accomplish the critical task of refolding or eliminating damaged and aggregated proteins. Here we investigate cooperation between the Lon protease and the IbpA and IbpB small heat-shock protein (sHSP) holdases in the model organism, *Escherichia coli*.

sHSPs are molecular chaperones that bind unfolded proteins and prevent their irreversible aggregation. sHSPs contain a central α-crystallin domain flanked by variable N- and C-terminal tails. These tails are responsible for the higher-order oligomerization, and therefore the chaperone functions, of sHSPs. The *E. coli* genome contains two sHSPs, *ibpA* and *ibpB*. We find that IbpA and IbpB are substrates of the Lon protease when in their free form, and also when they are bound to unfolded client proteins *in vivo* and *in vitro*. Interestingly, unlike other known substrates of AAA+ proteases, IbpA and IbpB seem to be recognized through a structural feature of their conserved α-crystallin domain, rather than through peptide motifs near their N- or C-termini. Furthermore, we find that IbpB facilitates the degradation of IbpA both *in vivo* and *in vitro,* and that the mechanism by which IbpB stimulates IbpA degradation is most likely through directly interacting with IbpA and making IbpA a better substrate, rather than by activating Lon and making Lon better able to degrade IbpA.

Finally we investigate the importance of the degradation of Ibps that are bound to aggregated client proteins and find that degradation of client-bound Ibps by Lon facilitates the refolding of Ibp-bound clients. These data therefore uncover a previously undescribed connection between the proteolytic branch and the holdase branch of the protein quality-control network. Furthermore, this work demonstrates that in addition to being important for the degradation of damaged or misfolded proteins, proteolysis also has a novel role in the refolding of aggregated proteins.

Thesis supervisor: Tania A. Baker
Title: E. C. Whitehead Professor of Biology

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Chapter One

Introduction

The significance of protein quality-control

Almost all biological processes require proteins. By and large, proteins must adopt a specific fold in order to perform their functions. Unfolded proteins are not only non-functional, but can also form large aggregates (Sharma *et al.*, 2009). The term "protein quality-control" refers to the process whereby proteins that become unfolded are either actively refolded or destroyed (Dougan *et al.*, 2002a). Cells have evolved several mechanisms for effective and efficient protein quality-control. These mechanisms include the ability to disaggregate, refold, sequester, and degrade damaged and unfolded proteins, without negatively affecting properly folded proteins (Fig. 1.1) (Georgopoulos and Welch, 1993; Parsell and Lindquist, 1993; Wickner *et al.*, 1999).

The work in this thesis addresses questions related to how the different arms of the quality-control network communicate with one another. Specifically, I investigate the connections between the holdase arm (responsible for the binding and sequestration of damaged proteins) and the protease arm (responsible for the degradation of damaged proteins) of the protein quality-control network. Therefore, I will use the following sections to review our current understanding of the different arms of the protein quality-control network and of how the different arms communicate with one another. Although many of these chaperone systems also play a part in the initial folding of proteins, I will be focusing on their roles in refolding damaged proteins, and will be predominantly focusing on the protein quality-control network of the model organism, *Escherichia coli*.

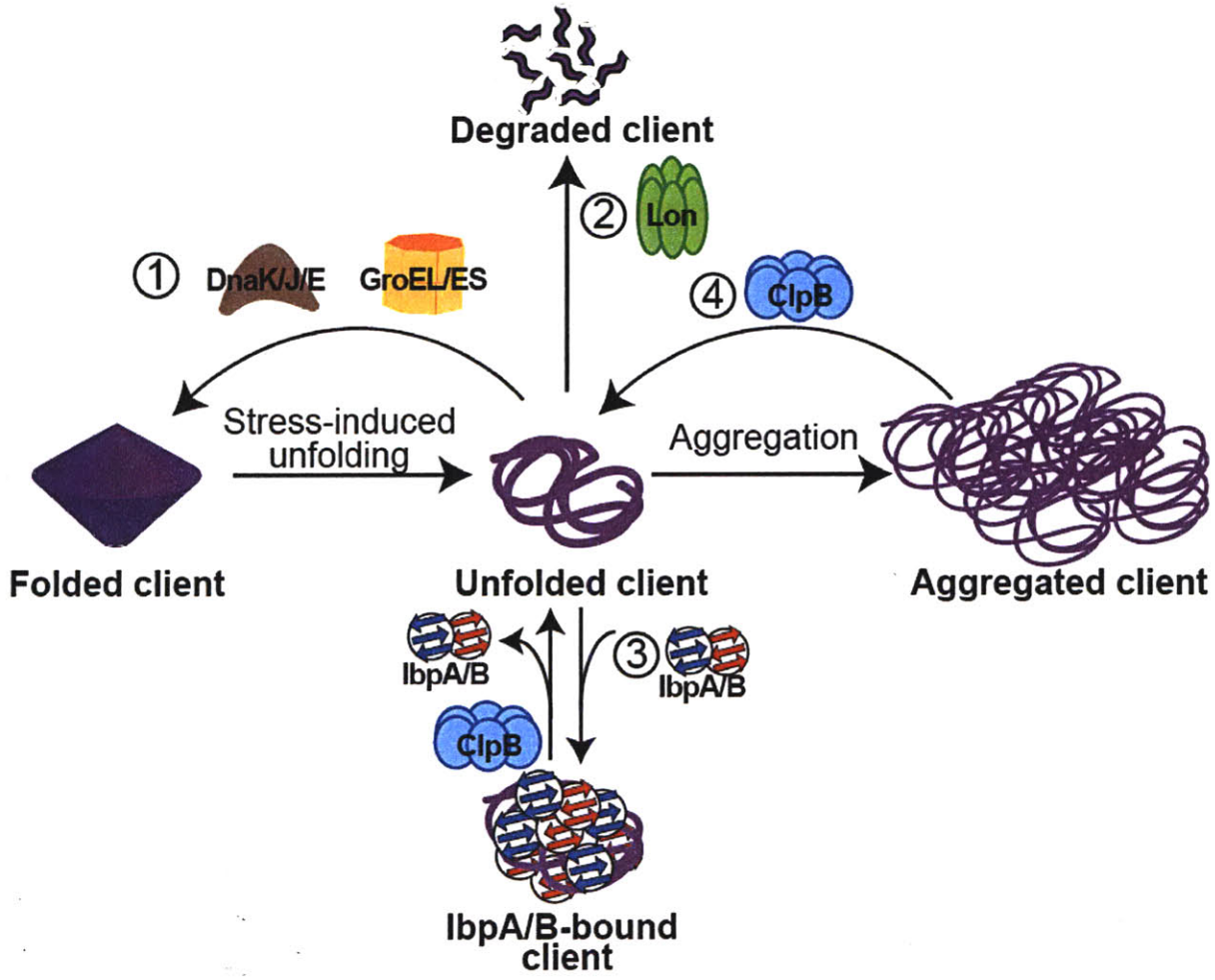

Figure 1.1: The *E. coli* protein quality-control network
The *E. coli* protein quality-control network is made up of several branches. (See text for details.) (1) Proteins that become unfolded due to environmental stress can be refolded by the refolding chaperones such as the GroEL/ES chaperones or the DnaK/DnaJ/GrpE (DnaKJE) chaperone system. (2) Unfolded proteins can also be degraded by Lon. (3) The small heat-shock proteins IbpA and IbpB bind unfolded proteins and protect them from irreversible aggregation until they can be refolded (pathway (1)). (4) Unfolded proteins that become aggregated can be disaggregated by the ClpB disaggregase.

Refolding Chaperones

GroEL/ES (Hsp60)

The GroEL/ES system is one of the two main refolding chaperone systems in *E. coli* (Fig. 1.1(1)) (Georgopoulos and Welch, 1993). The ability to refold unfolded proteins is critical to *E. coli*, as reflected by the fact that *groEL* and *groES* are essential genes (Fayet *et al.*, 1989). GroEL is an open, barrel-shaped double-ring 14-mer, while GroES is a ring-shaped 7-mer and forms the "cap" on the GroEL barrel (Boisvert *et al.*, 1996; Hunt *et al.*, 1996; Xu *et al.*, 1997). The mechanism of protein-folding by GroEL/ES has been extensively investigated. Current data show that protein-folding by GroEL/ES is accomplished by two distinct pathways (Horwich *et al.*, 2007).

One folding pathway involves sequestering unfolded proteins within the barrel of GroEL, which is then capped with the GroES co-chaperone (Walter, 2002). Sequential rounds of ATP hydrolysis cause conformational changes that lead to the binding or release of the GroES cap, which cause the GroEL client protein to be encapsulated or ejected (Roseman *et al.*, 1996; Thirumalai and Lorimer, 2001). Close confinement within the GroEL chamber, as well as the charged nature of the amino acid residues lining the inside of the compartment, are thought to facilitate the folding of client proteins (Tang *et al.*, 2006; Tang *et al.*, 2008). This mechanism is thus only useful for proteins that will fit into the barrel of GroEL, which include proteins that are smaller than 60 kDa. A second pathway involves the binding of client proteins to GroEL, but not encapsulation. This pathway operates on yeast mitochondrial aconitase, an 82 kDa enzyme which requires

yeast GroEL to fold, but which is too big to be encapsulated within GroEL (Chaudhuri *et al.*, 2001).

The DnaK system (Hsp70)

The second main refolding chaperone system in *E. coli* is known as the DnaK system, and is comprised of DnaK along with its co-chaperones DnaJ and GrpE (Mayer and Bukau, 2005). The DnaK system is thought to work by unfolding misfolded proteins, thereby giving these proteins another opportunity to refold into the correct conformation (Slepenkov and Witt, 2002). This mechanism therefore also works on proteins that are too large to be encapsulated in the GroEL chamber.

Like in the case of GroEL, binding to and release of client proteins from DnaK is controlled by the nucleotide-bound state of DnaK. Specifically, when DnaK is bound to ATP, it has low-affinity for client proteins, while ADP-bound DnaK has a high-affinity for client proteins (Mayer and Bukau, 2005). The nucleotide state of DnaK is in turn governed by the co-chaperones DnaJ and GrpE. DnaJ stimulates the ATPase activity of DnaK more than 1000-fold (Laufen *et al.*, 1999), while GrpE is a nucleotide exchange factor that binds ADP-bound DnaK, eliciting a conformational change that causes dissociation of ADP from DnaK (Harrison, 2004).

Interestingly, the genomes of many organisms, including *E. coli*, contain several copies of *dnaK* and *dnaJ* family members (Genevaux *et al.*, 2007). This fact suggested that different DnaK-DnaJ pairs might have specific functions in the cell, and indeed this

seems to be the case. For example, HscA and HscB (DnaK and DnaJ family members respectively) seem to be specifically required for the folding and maturation of iron-sulfur proteins (Barras *et al.*, 2005).

The ClpB disaggregase

For many years it was thought that protein aggregation was an irreversible process. This idea changed with the discovery of *HSP104* in *Saccharomyces cerevisiae*. *HSP104* was originally discovered as a gene required for thermotolerance in *S. cerevisiae*, and was later found to resolubilize a heat-aggregated luciferase fusion-protein *in vivo* (Parsell *et al.*, 1994; Sanchez and Lindquist, 1990). The *HSP104* homolog in *E. coli* is known as *clpB* (Doyle and Wickner, 2009). ClpB is a hexameric, barrel-shaped enzyme and is a member of the AAA+ (ATPases associated with various cellular activities) family of enzymes, all of which use ATP hydrolysis to perform various kinds of mechanical work (Hanson and Whiteheart, 2005; Lee *et al.*, 2003). ClpB is thought to disaggregate aggregated proteins by pulling on individual aggregated polypeptides and threading them through the pore of the ClpB barrel (Weibezahn *et al.*, 2004). The DnaK/DnaJ/GrpE system cooperates with ClpB in the disaggregation and refolding of aggregated proteins (Fig. 1.1(4), (1) (Motohashi *et al.*, 1999; Zietkiewicz *et al.*, 2004; Zietkiewicz *et al.*, 2006). This cooperation between the disaggregation branch and the refolding branch of the protein quality-control network is critical for the refolding of aggregated proteins and is discussed in more detail in a later section.

Holdases

The holdases are a family of chaperones that act to keep unfolded and damaged proteins in a refolding-competent state during times of environmental stress (Fig. 1.1(3)) (Narberhaus, 2002). In *E. coli* the holdases include the α-crystallin domain-containing small heat-shock proteins (sHSPs), and Hsp33 (Mchaourab *et al.*, 2009). The Hsp33 chaperone is a redox-regulated chaperone, and as such is normally inactive. However, under conditions of oxidative stress, two intramolecular disulfide bonds form, leading to dimerization (Kumsta and Jakob, 2009). This activates the holdase by creating a putative substrate binding site (Barbirz *et al.*, 2000; Graumann *et al.*, 2001). The sHSPs, on the other hand, are a much larger and more widely conserved family of proteins, and they will be the focus of the following sections.

sHSPs

sHSPs are a highly conserved family of proteins and have been found in every kingdom of life (Narberhaus, 2002). Interestingly, the small number of known organisms that do not contain sHSPs in their genomes are not necessarily the organisms with the smallest genomes (Kappé *et al.*, 2002). However, these organisms are all animal pathogens, suggesting that an important contributor to whether or not an organism encodes sHSPs may be how well their environment is controlled. sHSPs are characterized by having a central α-crystallin domain of about 100 residues, flanked by variable N- and C-terminal tail regions (Fig. 1.2A) (Sun and MacRae, 2005). Another characteristic of sHSPs is that they form large higher-order oligomeric assemblies, typically consisting of between 9 and 32+ subunits (Haslbeck *et al.*, 2005). Although poorly conserved, the N- and C-terminal tails of sHSPs have been shown to be essential for higher-order oligomer

formation, which in turn has been implicated in chaperone function (Leroux *et al.*, 1997; van de Klundert *et al.*, 1998).

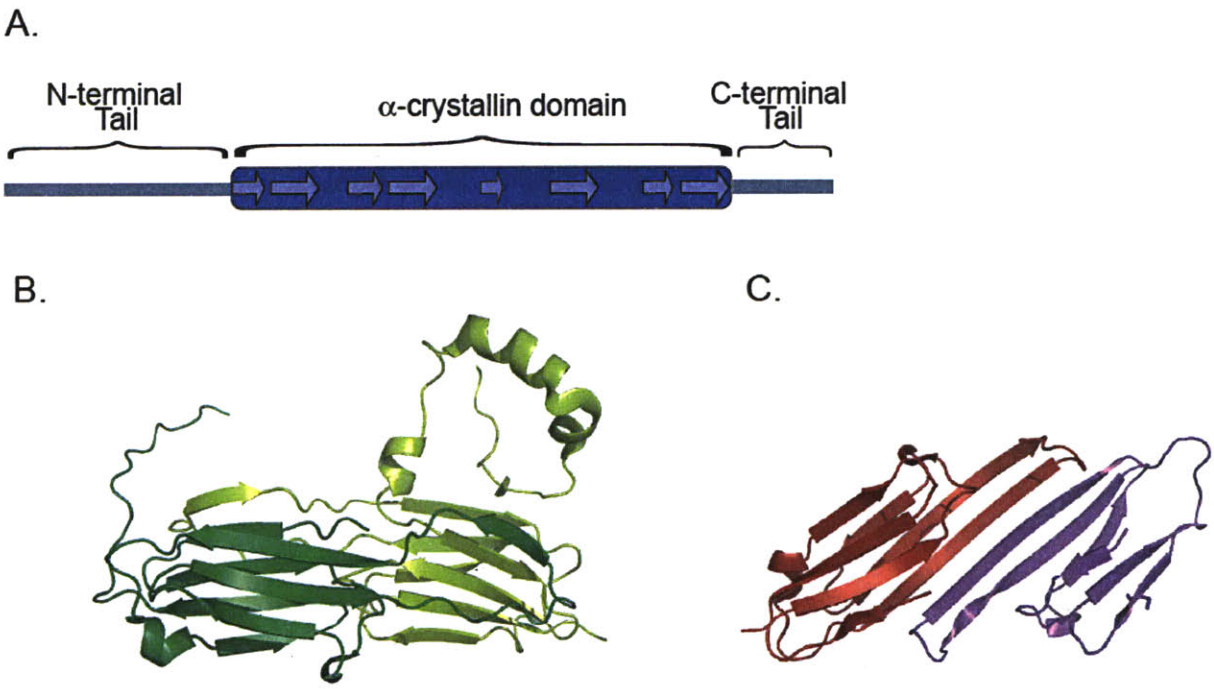

Figure 1.2: sHSPs—domain schematic and dimer organization.
(A) Schematic of sHSP monomers, showing the N- and C-terminal tails in gray, and the central α-crystallin domain in blue (Figure adapted from Mchaourab *et al.* 2009). Light blue arrows depict the 8 β-strands commonly found within the α-crystallin domain. (B) Crystal structure of the wheat sHSP 16.9 dimer (van Montfort *et al.*, 2001). The dimer is formed via strand exchange of a β-strand of one monomer contributing to a β-sheet of the neighboring monomer. (C) Crystal structure of the human αB-crystallin dimer (Bagnéris *et al.*, 2009). The dimer is formed by two monomers lining up side-to-side. (B) and (C) were created using PyMOL (DeLano, Warren L. The PyMOL Molecular Graphics System (2009) DeLano Scientific LLC, Palo Alto, California, USA. http://www.pymol.org).

Structure

As mentioned above, all sHSPs contain a central α-crystallin domain, named for the proteins in which this domain was originally found, the human eye lens α-crystallin proteins (Bloemendal *et al.*, 2004). The α-crystallin domain itself is made up of two β-sheets that fold in on themselves to form a β-sandwich (Mchaourab *et al.*, 2009). Although sHSPs form higher-order oligomers made up of 9-32+ subunits, the basic building block seems to be a dimer. However, even the dimer has multiple architectures. For example, in the wheat Hsp16.9, the dimer results from the strand exchange of one monomer forming part of the β-sheet of the second monomer and vice versa (Fig. 1.2B) (van Montfort *et al.*, 2001). However, in the case of the human sHSP αB-crystallin, the dimer forms from the two beta sandwiches packing side-to-side (Fig. 1.2C) (Bagnéris *et al.*, 2009). Furthermore, from the two solved crystal structures of oligomeric sHSPs, the architecture of the higher-order oligomer can also vary from species. *Methanocaldococcus jannashii* Hsp16.5 forms a spherical 24-mer, while the wheat Hsp16.9 forms a dodecamer formed from two stacked hexamers (Kim *et al.*, 1998; van Montfort *et al.*, 2001). Interestingly, although the N- and C-terminal tails of sHSPs are not required for dimer formation, they are involved in higher-order oligomerization. For example, in the case of wheat Hsp16.9, N-terminal tails from dimers on the top ring interact with the N-terminal tails from dimers on the bottom ring to hold the top and bottom rings together, while the C-terminal tails reach out and make stabilizing contacts with adjacent dimers in the same ring (van Montfort *et al.*, 2001).

Two other structural features of sHSPs are that subunits within the higher-order oligomers undergo subunit exchange, and that at higher temperatures, the higher-order complex dissociates into smaller subunits (Bova *et al.*, 2000; Haslbeck *et al.*, 1999). Oligomer rearrangement and subunit exchange are attractive mechanisms for how sHSPs could interact with unfolded client proteins. However, the idea that subunit exchange and oligomer dissolution are required for the chaperone function of sHSPs is controversial. While there is often a correlation between the dissociation of higher order complexes and the ability of sHSPs to perform chaperone functions *in vitro*, it has also been demonstrated that cross-linked sHSPs that do not dissociate at higher temperatures and do not exchange subunits are still functional chaperones (Benesch *et al.*, 2008; Franzmann *et al.*, 2005; Haslbeck *et al.*, 1999; Shashidharamurthy *et al.*, 2005).

Interactions with client proteins

In the absence of high-resolution structures of sHSPs bound to client proteins, the mode of interaction between sHSPs and their client proteins is still an open question. Cross-linking studies have demonstrated that the N-terminal tails of sHSPs are involved in the interactions between sHSPs and their client proteins (Jaya *et al.*, 2009). Furthermore, hydrophobic patches on sHSPs have been shown to be critical for chaperone function, presumably for binding to unfolded client proteins (Lee *et al.*, 1997). Interestingly, a study using hydrogen-deuterium exchange monitored by mass spectrometry demonstrated that the secondary structure of sHSPs is not altered by being bound to client proteins (Cheng *et al.*, 2008). Furthermore, there do not seem to be any solvent-

protected stable interfaces between sHSPs and client proteins (Cheng *et al.*, 2008). Rather, it seems that sHSPs interact with their clients dynamically.

E. coli *sHSPs*

The *E. coli* genome contains two sHSPs, *ibpA* and *ibpB*, which are transcribed from the same operon and whose amino acid sequences are close to 50% identical (Allen *et al.*, 1992). The Ibps were initially discovered as proteins that were up-regulated in *E. coli* during the heterologous over-expression of a variety of human and bovine proteins (Allen *et al.*, 1992). In this original study, the Ibps were found in inclusion bodies with the heterologously over-expressed proteins, and were consequently named inclusion-body binding proteins A and B. IbpA and IbpB were later reported to bind endogenous aggregated proteins based on the observation that upon heat-shock, the Ibps changed from co-fractionating with the outer-membrane, to co-fractionating with the insoluble protein fraction (Laskowska *et al.*, 1996).

Regulation of IbpA and IbpB abundance

The *ibp* operon is under transcriptional control of the heat-shock sigma-factor RpoH/σ^{32}, although there is also evidence that the *ibp* transcript contains an internal RpoN (a sigma factor for genes involved in nitrogen assimilation) binding-site, upstream of the *ibpB* open reading frame (Allen *et al.*, 1992; Kuczynska-Wisnik *et al.*, 2001; Reitzer and Schneider, 2001). Consistent with the idea that the *ibp* operon is under the control of a heat-shock sigma-factor, *ibpA* and *ibpB* transcripts are up-regulated 300-fold upon heat-shock (Richmond *et al.*, 1999). Additionally, the *ibp* mRNA seems to be an example of a

transcript that is controlled by a ROSE (repression of heat-shock gene expression) element, also known as an RNA thermometer (Waldminghaus *et al.*, 2009). The ROSE element is a temperature-sensitive RNA structure in the 5'UTR of certain mRNAs that inhibits the translation of these mRNAs at lower temperatures, but which "melts" at heat-shock temperatures, allowing for ribosome-binding and translation of the message (Nocker *et al.*, 2001).

Ibp mutant phenotypes and activities

Since their initial discovery, the Ibps have been found to be members of the sHSP family, as they contained predicted α-crystallin domains. Phenotypically, *Δibp* strains show reduced viability after extreme heat-shock compared to wild-type strains (Kuczynska-Wisnik *et al.*, 2002; Thomas and Baneyx, 1998). *Δibp* strains also require more time to resolubilize their aggregated proteins after heat-shock treatment (Mogk *et al.*, 2003a). Furthermore, cells over-expressing IbpA and IbpB have increased tolerance for heat-shock treatments and superoxide stress (Kitagawa *et al.*, 2000). More recently the Ibps have been implicated in resistance to copper-induced oxidative stress, and have even been shown to be important for biofilm formation (Kuczynska-Wisnik *et al.*, 2009; Matuszewska *et al.*, 2008).

Like other sHSPs, the Ibps were found *in vitro* to be competent to maintain aggregation-prone proteins in a refolding-competent state (Kitagawa *et al.*, 2002; Matuszewska *et al.*, 2005). Interestingly, despite the fact that their amino acid sequences are close to 50% identical, IbpA and IbpB seem to have distinct roles in protein quality-control. For

example, IbpA alone is competent to keep the model client protein luciferase in morphologically smaller aggregates, whereas IbpB alone does not have this activity (Ratajczak *et al.*, 2009). However IbpB is required for the downstream refolding of luciferase by the ClpB and DnaKJE systems (Ratajczak *et al.*, 2009). Consistent with the idea that the predominant role of IbpA is to interact with client proteins whereas the predominant role of IbpB is to interact with the refolding machinery, a global protein interaction study in *E. coli* found that IbpA interacted with many more proteins than did IbpB (Butland *et al.*, 2005).

Nevertheless, other *in vitro* studies have shown that IbpB alone is sufficient to suppress the aggregation of the aggregation-prone enzymes citrate synthase (CS), alcohol dehydrogenase (AD), and malate dehydrogenase (MDH) (Shearstone and Baneyx, 1999; Veinger *et al.*, 1998). Furthermore, it was demonstrated that GroES/EL chaperones are required downstream of the DnaKJE system for optimal refolding of IbpB-bound MDH (Veinger *et al.*, 1998). At present it is unclear whether this discrepancy between the ability of IbpB alone to suppress CS, AD, and MDH aggregation, and IbpB's inability to suppress luciferase aggregation is due to a difference in experimental setup or due to intrinsic differences between different client proteins. Nevertheless, how different branches of the protein quality-control network communicate with each other is a key question in the protein quality-control field, and one that is the main focus of the work in this thesis.

Proteases

The *E. coli* genome encodes a number of different proteases that exist in various compartments of the cell and have differing functions (Gottesman, 1996). The proteases most important for protein quality-control are Lon and ClpAP, both members of the AAA+ family of enzymes (Dougan *et al.*, 2002b; Van Melderen and Aertsen, 2009). As mentioned previously, AAA+ enzymes are characterized by the fact that they all use the energy of ATP hydrolysis to perform mechanical work (Hanson and Whiteheart, 2005). In the case of the AAA+ proteases, this work is the unfolding and translocation of substrate proteins through the narrow axial pore of the enzyme to where the proteolytic active sites reside (Fig. 1.3) (Baker and Sauer, 2006). ClpAP, along with its adaptor protein ClpS, has been implicated in the degradation of aggregated proteins *in vitro* (Dougan *et al.*, 2002b), whereas Lon has been implicated in protein quality-control through a variety of genetic and biochemical experiments. The results of these experiments are discussed further in the following sections.

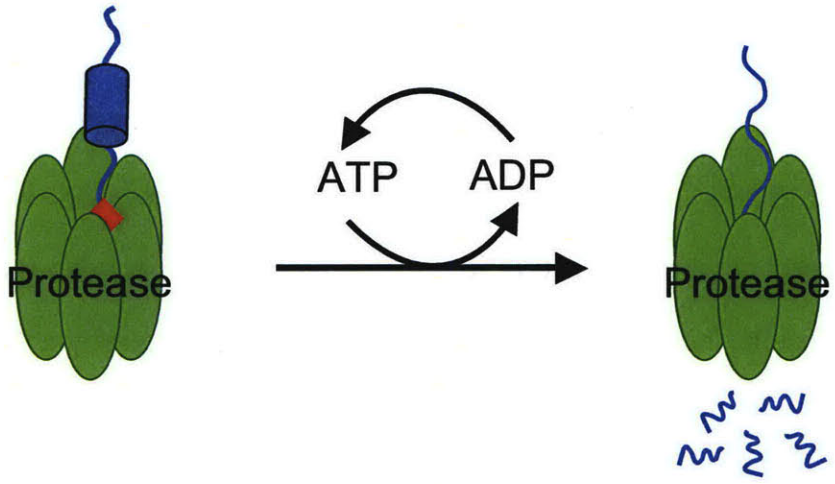

Figure 1.3: Degradation of substrates by AAA+ proteases
AAA+ proteases bind substrates most often through short sequence tags near the N- or C-termini of the substrate, and then unfold, translocate, and degrade their substrates through multiple rounds of ATP hydrolysis.

Lon

lon was originally identified in *E. coli* in a genetic screen for genes involved in resistance to UV damage (Adler and Hardigree, 1964b). In addition to being hypersensitive to UV irradiation, *lon*$^-$ mutants also exhibited an unusually *long* cell morphology, the phenotype for which the *lon* gene is named (Adler and Hardigree, 1964a; HOWARD-FLANDERS *et al.*, 1964). Lon has been implicated in a number of important processes in *E. coli* including cell division, resistance to oxidative and UV stress, and the degradation of abnormal and damaged proteins (Tsilibaris *et al.*, 2006; Van Melderen and Aertsen, 2009). The role of Lon in these processes is discussed in the following sections.

Structure

Lon is a homo-hexamer composed of 87 kDa subunits (Fig. 1.4A) (Botos *et al.*, 2004b). Each subunit is made up of three domains (Fig. 1.4B). The N-terminal (N) domain is thought to be important for substrate binding. This conclusion came from the isolation of a point mutant (E240K) in a predicted coiled-coil region within the N domain that eliminated the ability of Lon to degrade one of its *in vivo* substrates, RcsA, but that was still able to degrade another substrate, SulA (Ebel *et al.*, 1999). The first 119 amino acids of the N domain have been crystallized, and adopt a novel fold (Li *et al.*, 2005), although the significance of this structure for substrate binding, if any, is unclear.

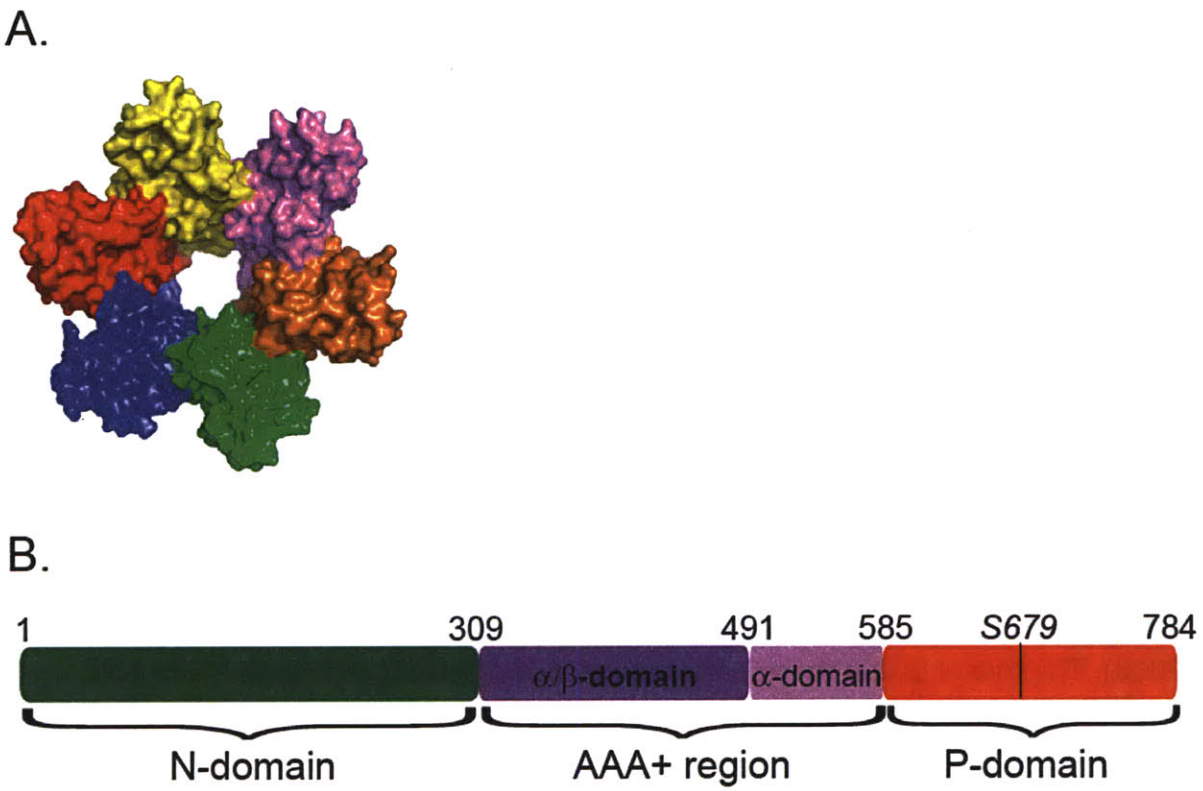

Figure 1.4: Lon structure and domain organization
(A) Crystal structure of the Lon P-domain depicting Lon's hexameric structure (Botos *et al.*, 2004b). Figure created using PyMOL. (B) Domain structure of *E. coli* Lon detailing the boundaries of the N-domain, the AAA+ region, and the P domain. The catalytic serine S679 is also depicted. (Adapted from Rotanova *et al.* 2006).

Like all AAA+ homology regions, Lon's AAA+ homology region is divided into two domains, the α/β-domain and the α-domain (Rotanova *et al.*, 2006). The α/β-domain contains the Walker A, Walker B and sensor-1 motifs, all involved in nucleotide binding and hydrolysis (Hanson and Whiteheart, 2005; Neuwald *et al.*, 1999). The α-domain contains the sensor-2 motif, which is also involved in ATP hydrolysis (Hanson and Whiteheart, 2005; Neuwald *et al.*, 1999). The crystal structure of the α-domain has been

solved and adopts the conserved fold seen with many other α-domains (Botos *et al.*, 2004a).

The C-terminal proteolytic (P) domain contains the proteolytic active site of the enzyme, S679, which is part of a serine-lysine catalytic dyad (Rotanova *et al.*, 2006). The presence of the catalytic dyad was confirmed by the crystal structure of the P domain, as was the hexameric structure of Lon (Botos *et al.*, 2004b). The P domain alone is not able to degrade proteins, but is able to degrade small peptides (Botos *et al.*, 2004b; Rasulova *et al.*, 1998).

E. coli *Lon substrates and substrate recognition*

To date, the *in vivo* degradation tags that have been described for Lon seem to be specific for each Lon substrate. This is in contrast to other AAA+ proteases such as ClpXP and ClpAP, both of which recognize the ssrA tag (Gottesman *et al.*, 1998), a tag that is appended onto all polypeptide products of stalled translation, targeting them for degradation (Karzai *et al.*, 2000). Due to this ability to recognize the ssrA tag, ClpXP and ClpAP degrade ssrA-tagged proteins. Establishing the degradation determinants of protease substrates is valuable, as knowing the specific determinants that proteases use to recognize their substrates can facilitate the identification of new substrates. Furthermore, this knowledge can also be used to design experiments to probe the mechanism by which proteases recognize their substrates.

Despite the fact that there is no known general recognition motif for Lon substrates, there are several well-characterized Lon substrates with known specific degradation determinants. In addition, Lon is known to degrade unfolded and damaged proteins *in*

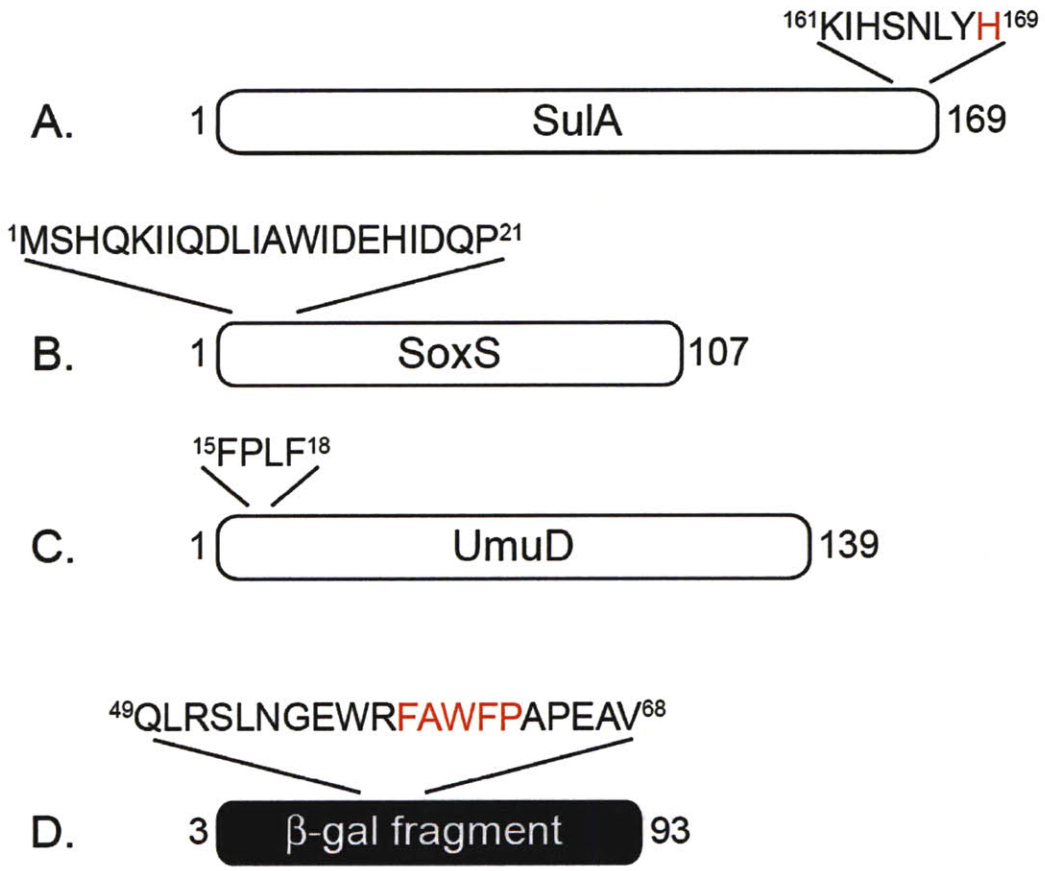

Figure 1.5: Summary of selected Lon degradation signals
(A) SulA: The last 8 amino acids, and especially the very C-terminal histidine has been implicated in SulA degradation (Ishii and Amano, 2001). (B) SoxS: The first 17 amino acids of SoxS are important for recognition by Lon (Shah and Wolf, 2006a). (C) UmuD: Residues 15-18 are thought to be the most important for Lon recognition (Gonzalez *et al.*, 1998). (D) Unfolded proteins: Unfolded proteins are recognized by Lon through patches of hydrophobic residues (Gur and Sauer, 2008b). As an example, for the degradation of a β-galactosidase fragment (residues 3-93), a centrally located 20 amino acids containing an FAWFP motif at its core is critical for the degradation of this fragment.

vivo and *in vitro*. The rules that are thought to govern substrate recognition or degradation in these cases are discussed in the following sections (Fig. 1.5).

SulA

SulA was one of the first Lon substrates to be described. In fact *sulA* was named for the observation that mutations in this gene suppressed the UV-sensitivity exhibited by *lon*$^-$ mutants (suppressor of *lon* A) (Gayda *et al.*, 1976). It was later discovered that SulA is an inhibitor of cell septation, and that the reason that *lon*$^-$ cells exhibit an elongated morphology is because SulA is a Lon substrate (Huisman *et al.*, 1984; Mizusawa and Gottesman, 1983). In the absence of Lon, SulA accumulates, and therefore cells grow without septation.

Interestingly, investigation of the degradation determinants of SulA revealed that the histidine at the very C-terminus of SulA is critical for its degradation by Lon, and that mutating this single amino acid was sufficient to abrogate SulA degradation (Fig. 1.5A) (Ishii and Amano, 2001). Furthermore, although truncating the C-terminal 8 amino acids of SulA also abrogated SulA degradation, appending a histidine to the new C-terminus was again sufficient to cause SulA to be degraded by Lon (Ishii and Amano, 2001). Therefore this seems to be an unusual case where in the context of a specific substrate, a single amino acid is able to confer the ability to be degraded on this substrate.

SoxS

SoxS is a transcriptional activator of a variety of genes, all involved in defending cells against reactive oxygen species (Storz and Imlay, 1999). *In vivo* SoxS is degraded rapidly, having a half-life of about 2 minutes. Degradation of SoxS *in vivo* has been attributed to Lon (Griffith *et al.*, 2004). The first 17 amino acids of SoxS are important for the degradation of SoxS by Lon (Fig. 1.5B), and appending the first 21 amino acids of SoxS (MSHQKIIQDLIAWIDEHIDQP) onto GFP is sufficient to convert GFP into a Lon substrate (Shah and Wolf, 2006a). Intriguingly, binding of SoxS to promoter regions on DNA inhibited the Lon-dependent degradation of SoxS (Shah and Wolf, 2006b).

UmuD

UmuD is part of the SOS regulon, a group of genes that is expressed in response to DNA damage (Janion, 2008). Once it is activated, UmuD' is part of the DNA pol V holoenzyme that bypasses otherwise replication-blocking lesions (Gonzalez and Woodgate, 2002). Normally UmuD is degraded by Lon and is therefore inactive (Frank *et al.*, 1996). However, upon exposure to DNA damaging agents UmuD is cleaved by RecA, yielding UmuD' which is the active form and which is no longer a Lon substrate (Gonzalez *et al.*, 1998; Shinagawa *et al.*, 1988).

Alanine stretch mutagenesis experiments found that the FPLF motif (UmuD residues 15-18) were the most important residues for Lon recognition of UmuD (Fig. 1.5C), although the FPSP motif at residues 26-29 also played a role (Gonzalez *et al.*, 1998). Appending residues 1-40 of UmuD onto phleomycin resistance protein (PRP) was sufficient to convert PRP into a Lon substrate, and mutating the residues noted above

abrogated this degradation (Gonzalez *et al.*, 1998). The degradation of this chimeric construct therefore implicates specific sequence motifs within the first 40 residues of UmuD as Lon degradation determinants (Gonzalez *et al.*, 1998).

Unfolded proteins

It has been known for many years that Lon is involved in the degradation of damaged and misfolded proteins. For example, Lon has been linked to the degradation of a β-galactosidase nonsense fragment, puromycyl peptides and canavanine-containing proteins *in vivo* (Kowit and Goldberg, 1977; Maurizi *et al.*, 1985). More recently it has been demonstrated that *lon*$^-$ cells accumulate more aggregated proteins than do cells lacking other proteases such as ClpP, implicating Lon in the degradation of aggregates or pre-aggregated misfolded proteins (Rosen *et al.*, 2002).

Recently it was shown that Lon recognizes unfolded proteins by recognizing exposed peptides with high surface-burial scores (Gur and Sauer, 2008b). A surface-burial score is a score that can be given to a peptide sequence that reflects how likely that sequence is to be buried versus solvent exposed, within the context of a full-length protein (Rose *et al.*, 1985). By scanning the sequence of a fragment of β-galactosidase that is robustly degraded by Lon and looking for regions with high surface-burial scores, it was found that there was an excellent correlation between the magnitude of the surface-burial score of a given peptide segment, and the rate at which these peptides were degraded by Lon *in vitro* (Gur and Sauer, 2008b). Furthermore, when the highest scoring peptide (Fig. 1.5D) was appended onto the I27 domain of titin, a tightly folded model protein, the

I27 domain of titin was now able to be degraded by Lon (Gur and Sauer, 2008b). This work suggests that the way that Lon recognizes unfolded proteins is by recognizing exposed regions that would normally be buried in the hydrophobic core of the protein.

As described here, Lon uses a variety of different methods to recognize substrates. Given this variety, we were particularly interested in investigating the mechanism by which Lon recognizes the Ibps. We found that Lon seems to recognize the Ibps using a completely different mechanism from those outlined above, as will be demonstrated in Chapter 2.

Mitochondrial Lon

In eukaryotes, Lon is found in the mitochondrial matrix where it is responsible for the degradation of specific proteins, including the unassembled subunits of the mitochondrial processing peptidase, which is responsible for cleaving the mitochondrial targeting sequences from proteins (Ondrovicová *et al.*, 2005). Mitochondrial Lon has also been shown to be important for the replication of mitochondrial DNA, for chaperoning the assembly of inner membrane complexes, and for the degradation of oxidatively damaged proteins (Bota and Davies, 2002; Liu *et al.*, 2004; Rep *et al.*, 1996).

Recently Lon has been implicated in cellular aging. Cellular aging correlates remarkably well with a decline in Lon expression and function (Bota *et al.*, 2002; Lee *et al.*, 1999). Perhaps most intriguing is work that showed that over-expression of Lon in *Podospora*

anserina, a fungal model for aging, increased the lifespan of this organism by almost two-fold (Luce and Osiewacz, 2009). This effect was dependent on the proteolytic activity of Lon, as over-expression of a proteolytically inactive mutant variant of Lon did not increase *P. anserina* life span. The involvement of Lon in the aging process is thought to be the result of the ability of Lon to preferentially degrade oxidatively damaged proteins (Luce and Osiewacz, 2009). And indeed bovine mitochondrial Lon was shown to degrade oxidized aconitase but not undamaged aconitase *in vitro* (Bota and Davies, 2002). Furthermore, in the *P. anserina* study, cells over-expressing Lon were shown to contain a reduced amount of carboxymethylated and carbonylated proteins, which are both hallmarks of oxidative stress (Luce and Osiewacz, 2009; Nyström, 2005). These results suggest that the age-related pathology that results from the accumulation of oxidatively damaged proteins may be attributed to the diminished levels and activity of mitochondrial Lon.

Interactions between different branches of the protein quality-control network

As depicted in Figure 1.1, damaged or aggregated proteins can require multiple branches of the protein quality-control network to be refolded. Consistent with this idea, work from a number of labs has established that there is cooperation between certain branches of the protein quality-control network (see below). This section will summarize our current understanding of which branches of the protein quality-control network communicate with each other, and how this communication facilitates protein quality-control.

Communication between the ClpB disaggregase and the refolding chaperones

ClpB provides an excellent example of how different members of the protein quality-control network communicate with each other, because ClpB disaggregates aggregated proteins, but cannot refold them (Goloubinoff *et al.*, 1999). Genetic and biochemical experiments demonstrate that the DnaK system cooperates with ClpB to reverse aggregation. In a *ΔrpoH* strain, (a heat-shock sensitized mutant back ground) heterologous expression of both the DnaK system and ClpB is required for efficient protein disaggregation (Tomoyasu *et al.*, 2001). Furthermore, the DnaK system and ClpB act synergistically to reactivate aggregated GFP *in vitro* (Doyle *et al.*, 2007).

Communication between proteases and the refolding chaperones

There is also evidence that the proteolytic branch and the refolding branch of the protein quality-control network communicate with each other. One of the first pieces of evidence for this communication came from the observation that DnaK, GrpE, GroEL, and Lon co-immunoprecipitate with an unfolded abnormal fusion protein that contained parts of the cro repressor, protein A and β-galactosidase (Sherman and Goldberg, 1991). Furthermore, Lon-mediated *in vivo* degradation of a mutant form of alkaline phosphatase (phoA61) is almost completely abolished in the absence of DnaK (MYu and Goldberg, 1992). This observation suggests that unfolding of client proteins by DnaK might facilitate their degradation by Lon.

There is also evidence that proteases can compensate for decreased refolding capacity. Genetic experiments reveal that when DnaK is depleted under heat-shock conditions,

ClpXP and Lon become essential for viability, suggesting that the proteolytic machinery and the DnaK system act synergistically under these conditions to clear the cell of misfolded proteins (Tomoyasu *et al.*, 2001). Therefore, the proteolytic branch and the refolding branch of the protein quality-control network seem to cooperate in at least two distinct ways. First, refolding or unfolding of abnormal proteins seems to facilitate their degradation. Second, refolding chaperones and proteases act synergistically to maintain the minimal amount of protein homeostasis required for viability.

Communication between sHSP holdases, ClpB, and the refolding chaperones

Finally, the sHSP holdases must cooperate with other branches of the protein quality-control network, as they keep client proteins in a refolding-competent state until the clients can be refolded by the refolding chaperones. Moreover, since Ibps co-aggregate with client proteins (Jiao *et al.*, 2005a), it might be expected that the Ibps would cooperate with both ClpB and the refolding chaperones, and indeed this is the case. Specifically, sHSPs cooperate with ClpB to reverse protein aggregation *in vivo* (Mogk *et al.*, 2003a). Furthermore, sHSPs cooperate with ClpB and the DnaK system, or alternatively with the DnaK system and GroEL/ES, to refold model client proteins *in vitro* (Mogk *et al.*, 2003b; Ratajczak *et al.*, 2009; Veinger *et al.*, 1998). However, unlike the refolding chaperones, to date the Ibps have not been shown to cooperate with the proteolytic branch of the protein quality-control network.

Thesis Overview

This thesis focuses on three main projects. First I determined that IbpA and IbpB are substrates of the Lon protease and I conducted structure-function studies to identify the degradation determinants of the Ibps. Second, I demonstrated that IbpA and IbpB cooperate in their degradation by Lon, and I studied how this cooperation takes place. Lastly I have begun to investigate the biological significance of this previously undescribed interaction between the Ibps and Lon, two of the major players in protein quality-control. This work therefore establishes a link between the holdase branch and the proteolytic branch of the protein quality-control network, suggests a biological explanation for Ibp proteolysis, and provides a framework for future studies investigating cooperation between the Ibps and Lon.

Chapter Two

The IbpA and IbpB small heat-shock proteins are substrates of the AAA+ Lon protease

This chapter has been submitted for publication as "Bissonnette, S.A., Rivera-Rivera, I., Sauer, R.T., Baker, T.A. (2009)". I.R.R. conducted the experiments in Figure 2.2. S.A.B. carried out the remaining experiments. S.A.B., R.T.S. and T.A.B. contributed to experimental design and preparation of the manuscript.

Abstract

Small heat-shock proteins (sHSPs) are a widely conserved family of molecular chaperones, all containing a conserved α-crystallin domain flanked by variable N- and C-terminal tails. We report that IbpA and IbpB, the sHSPs of *Escherichia coli,* are substrates for the AAA+ Lon protease. This ATP-fueled enzyme degraded purified IbpA substantially more slowly than purified IbpB, and we demonstrate that this disparity is a consequence of differences in maximal Lon degradation rates and not in substrate affinity. Interestingly, however, IbpB stimulated Lon degradation of IbpA both *in vitro* and *in vivo*. Furthermore, although the variable N- and C-terminal tails of the Ibps were dispensable for proteolytic recognition, these tails contain critical determinants that control the maximal rate of Lon degradation. Finally, we show that *E. coli* Lon degrades variants of human α-crystallin, indicating that Lon recognizes conserved determinants in the folded α-crystallin domain itself. These results suggest a novel mode for Lon substrate recognition and provide a highly suggestive link between the degradation and sHSP branches of the protein quality-control network.

Introduction

Organisms constantly combat environmental insults, which can denature proteins, destroy molecular function, and rapidly result in protein aggregates. Under these circumstances, quality-control networks in the cell attempt to refold damaged proteins (Feder and Hofmann, 1999; Visick and Clarke, 1995). In *Escherichia coli,* the quality-control network includes IbpA and IbpB, which are members of the small heat-shock protein family (sHSPs), as well as the energy-dependent chaperones GroEL/GroES and DnaK/DnaJ/GrpE, and the ClpB disaggregase (Georgopoulos and Welch, 1993; Lund, 2001; Parsell and Lindquist, 1993). Lon and other AAA+ (ATPases associated with various cellular activities) proteases also play a major role in clearing misfolded and damaged proteins from the cell. Recent work demonstrates that certain branches of the quality-control network can cooperate to refold damaged proteins (Doyle *et al.*, 2007; Mogk *et al.*, 2003a; Mogk *et al.*, 2003b). Here we investigate a possible link between the protein degradation branch and the sHSP branch of the protein quality-control network.

The sHSPs are a family of ATP-independent molecular chaperones. Current studies indicate that sHSPs bind and function to protect misfolded proteins from irreversible aggregation (Laskowska *et al.*, 1996; Lee *et al.*, 1997; Narberhaus, 2002). All sHSPs contain a central conserved α-crystallin domain flanked by N- and C-terminal tails of variable length and amino acid composition (Haslbeck *et al.*, 2005). sHSPs exist as higher-order oligomers, a characteristic that is crucial for client-protein protection (Leroux *et al.*, 1997; van de Klundert *et al.*, 1998).

E. coli has two sHSPs, IbpA and IbpB, which are expressed from the same operon (Allen *et al.*, 1992). The Ibps are important for resistance to heat stress, as well as superoxide and copper-induced oxidative stress (Kitagawa *et al.*, 2000; Matuszewska *et al.*, 2008; Thomas and Baneyx, 1998). Furthermore, IbpA and IbpB interact with each other at elevated temperatures *in vitro* and cooperate to keep client proteins in a refolding-competent state (Matuszewska *et al.*, 2005). After thermal inactivation, for example, luciferase is reactivated more efficiently by ClpB and DnaK/J/E *in vitro* if both IbpA and IbpB are present during the initial thermal inactivation, than if only one of the Ibps is present (Ratajczak *et al.*, 2009). The Ibps also cooperate with ClpB and DnaK to reverse protein aggregation *in vivo* (Mogk *et al.*, 2003a; Veinger *et al.*, 1998). Thus, the Ibps interact functionally with the disaggregation and refolding machinery of the cell. However, to date, the Ibps have not been specifically linked to the protein-degradation machinery.

Lon is the principal protease responsible for the degradation of damaged and misfolded proteins in *E. coli* (Fredriksson *et al.*, 2005; Kowit and Goldberg, 1977). For example, Lon-deficient cells accumulate more aggregated proteins following heat-shock than cells missing other AAA+ proteases (Rosen *et al.*, 2002; Tomoyasu *et al.*, 2001). Recent work demonstrates that Lon can directly recognize misfolded substrates by binding to exposed hydrophobic regions, which would normally be buried in properly folded proteins (Gur and Sauer, 2008b), providing insight into one mode by which Lon recognizes substrates.

In this study, we report that IbpA and IbpB are themselves substrates for Lon, suggesting that there is functional cross-talk between the sHSPs and the protein degradation machinery. We further demonstrate that the rates of degradation of the Ibps are controlled by their N- and C-terminal tails. Unexpectedly, however, these tails are not required for Lon recognition. Rather, they influence the maximal speed of Lon proteolysis. Finally, we show that the presence of IbpB accelerates the rate of Lon degradation of IbpA both *in vitro* and *in vivo.* Our results therefore provide a previously unknown link between the sHSP and the protein-degradation branches of the quality-control network and further suggest a distinct method of substrate recognition by Lon.

Results

The *E. coli* sHSPs IbpA and IbpB are Lon substrates.

IbpA and IbpB were initially identified in a proteomic screen for substrates of *E. coli* Lon protease (E. Oakes and TAB, personal communication), using a strategy similar to previous screens for ClpXP substrates (Flynn *et al.*, 2003; Neher *et al.*, 2006). Briefly, Lon carrying an active-site mutation was used to trap substrates *in vivo* (Van Melderen and Gottesman, 1999), and the trapped substrates were identified by mass spectrometry. To determine directly whether IbpA and IbpB are substrates for Lon, we purified ^{35}S-labeled IbpA and IbpB and measured the rate at which Lon proteolysis generated acid-soluble radioactive peptides. Both proteins were degraded by Lon (Fig. 2.1B). However, despite the fact that IbpA and IbpB are nearly 50% identical at the sequence level (Fig. 2.1A), Lon degraded IbpB much faster than IbpA (Fig. 2.1B).

Table 2.1: Steady-state kinetic parameters for Lon degradation of the Ibps and the human α-crystallin proteins

Substrate	K_M (μM)	V_{max} (Substrate degraded min^{-1} Lon_6^{-1})
IbpA	18 ± 16	0.043 ± 0.018
IbpB	16 ± 2.7	0.60 ± 0.06
IbpA$^{\alpha}$	17 ± 4.7	0.47 ± 0.06
IbpB$^{\alpha}$	16 ± 2.4	0.19 ± 0.01
hαA-crystallin	50 ± 20	0.12 ± 0.05
hαB-crystallin	58 ± 21	0.06 ± 0.02
hαA$^{\alpha}$-crystallin	35 ± 8.9	2.5 ± 0.3
hαB$^{\alpha}$-crystallin	26 ± 12	3.8 ± 1.4

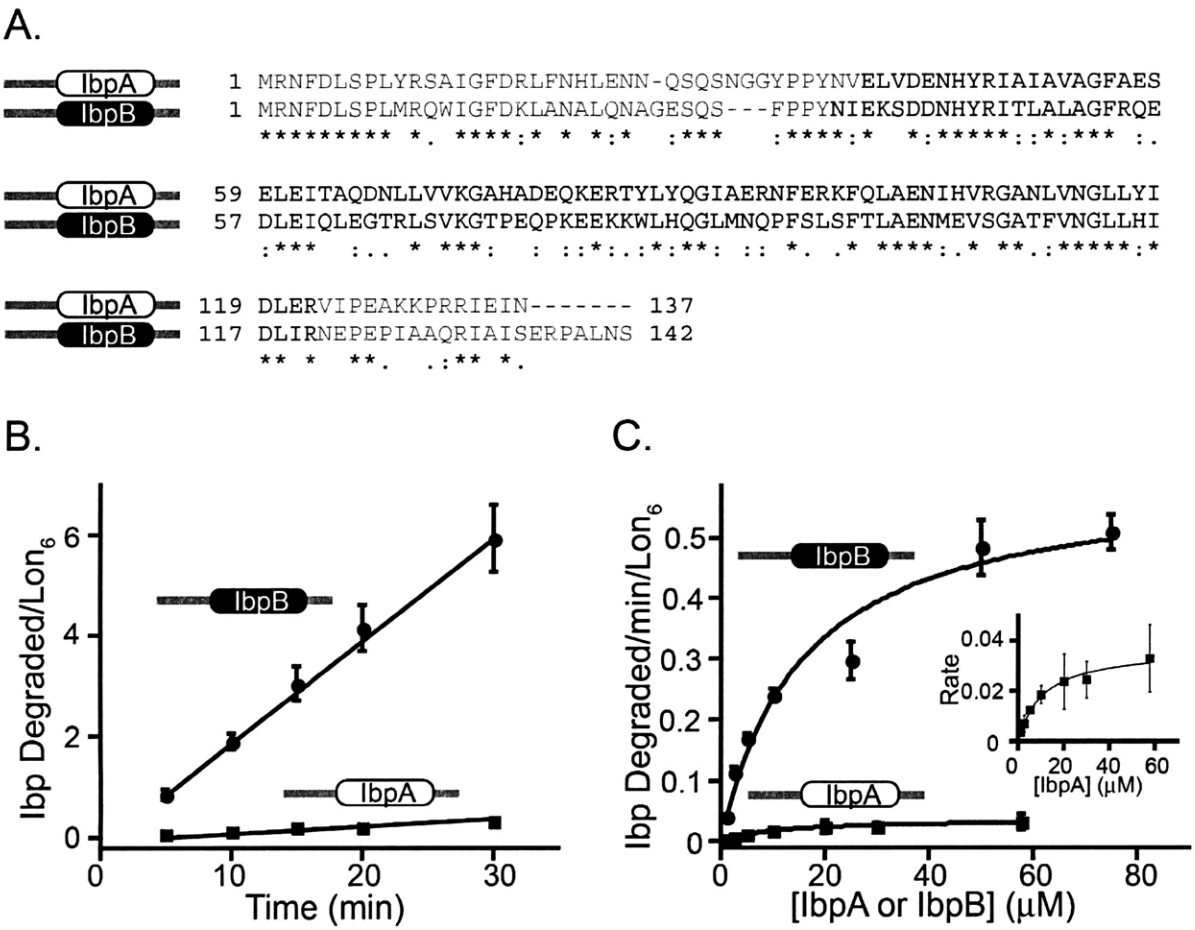

Figure 2.1: *E. coli* sHSPs IbpA and IbpB are Lon substrates.
(A) Sequence alignment of IbpA and IbpB generated by ClustalW2. Darker letters represent the conserved α-crystallin domain. Identical residues are denoted by (*), residues with the same size and hydropathy are denoted by (:), residues with the same size or hydropathy or denoted by (.). (B) Lon (600 nM hexamer) degradation of 5 μM IbpA (0.010 ± 0.001 min^{-1} Lon_6^{-1}) or 5 μM IbpB (0.20 ± 0.02 min^{-1} Lon_6^{-1}). (C) Substrate dependence of Lon degradation of IbpA (V_{max} = 0.043 ± 0.018 min^{-1} Lon_6^{-1}, K_M = 18 ± 16 μM) or IbpB (V_{max} = 0.60 ± 0.06 min^{-1} Lon_6^{-1}, K_M = 16 ± 2.7 μM). Degradation rates were measured from experiments like the one shown in panel B and were fit to the Michaelis-Menten equation. Error bars (± 1 SD) in this and all other figures were calculated from at least three independent experiments. The large error in the K_M for IbpA is due to the slow rate of degradation. Inset: The IbpA data are replotted on an expanded scale to show the curvature of the fitted line.

This difference in degradation rates could result from altered Lon recognition, which would be reflected in different K_M values for proteolysis. Alternatively (or in addition), Lon processing of IbpA and IbpB might differ at a downstream step, such as substrate unfolding or translocation, leading to differences in V_{max}. To distinguish between these possibilities, we determined steady-state rates of degradation of varying concentrations of IbpA and IbpB by Lon and fit these data to the Michaelis-Menten equation (Fig. 2.1C). Interestingly, the K_M values for Lon degradation of IbpA and IbpB were nearly identical (16-18 μM). However, the V_{max} for IbpB degradation was almost 15-fold higher than that of IbpA (Fig. 2.1C; Table 2.1). Thus, Lon recognizes IbpA and IbpB as substrates equally well but processes IbpB much faster than IbpA.

Ibp tails are not required for Lon recognition.

The degradation determinants of AAA+ protease substrates are often found near the very N- or C-terminus of the substrate (Flynn *et al.*, 2003; Gottesman *et al.*, 1998; Tobias *et al.*, 1991). Furthermore, the N- and C-terminal tails of sHSPs are known to be important for oligomerization and client-protein protection (Fernando and Heikkila, 2000; Jiao *et al.*, 2005b). To investigate the role of these terminal tails in Lon degradation of IbpA and IbpB, we constructed and purified Ibp variants lacking both tails and containing only the α-crystallin domain (referred to as $IbpA^{\alpha}$ and $IbpB^{\alpha}$). Intriguingly, at substrate concentrations of 5 μM, Lon degraded $IbpB^{\alpha}$ about a third as fast as IbpB but degraded $IbpA^{\alpha}$ 10-times faster than IbpA (Fig. 2.2A). The higher rate of degradation of $IbpA^{\alpha}$ than IbpA was not caused by a truncation-induced loss of protein secondary structure, as determined by circular-dichroism spectroscopy (data not shown). Thus,

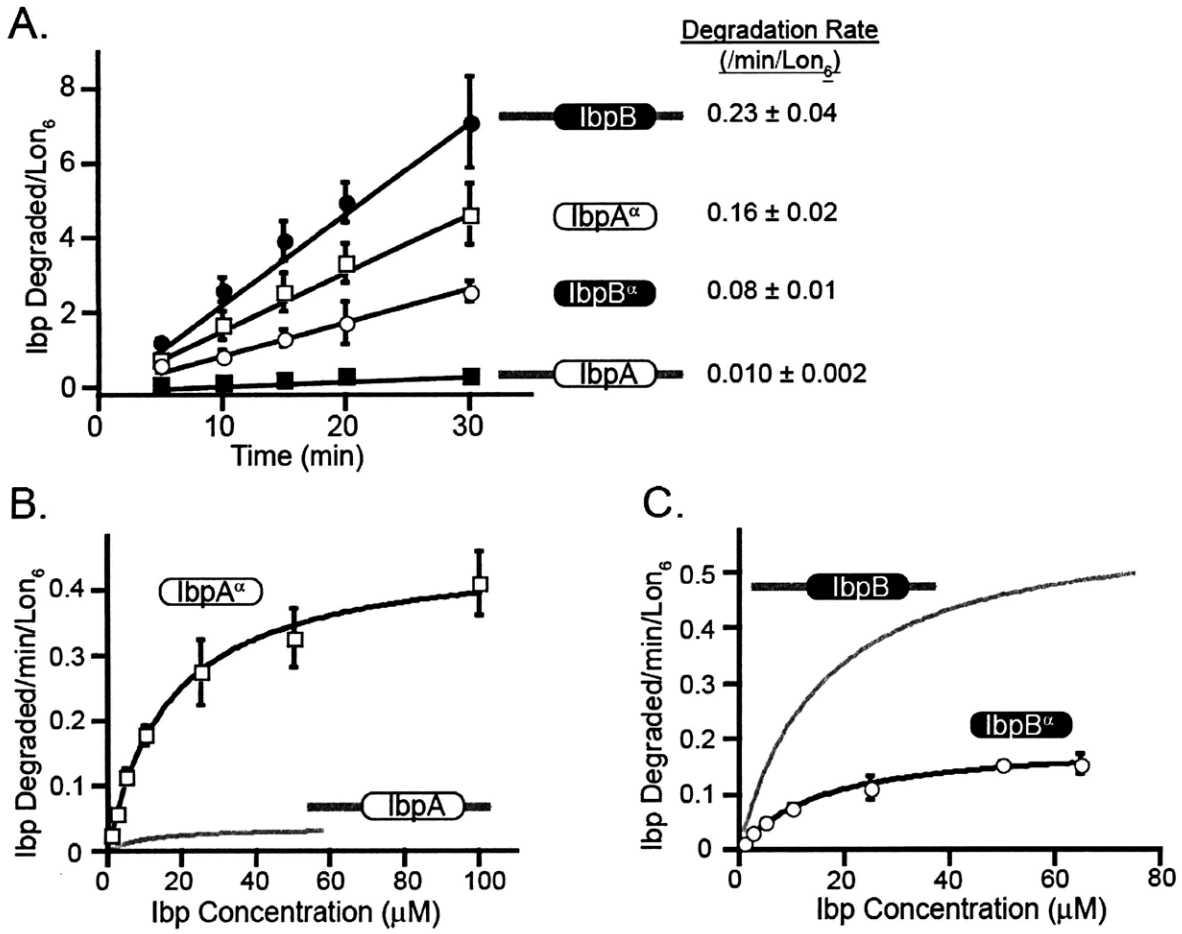

Figure 2.2: Lon degrades the isolated α-crystallin domains of IbpA and IbpB. (A) Degradation of 5 μM substrate by 600 nM Lon_6. IbpA (filled squares), IbpB (filled circles), $IbpA^{\alpha}$ (open squares), or $IbpB^{\alpha}$ (open circles). (B) Michaelis-Menten plot for $IbpA^{\alpha}$ (V_{max} = 0.47 ± 0.06 min^{-1} Lon_6^{-1}, K_M = 17 ± 4.7 μM). (C) Michaelis-Menten plot for $IbpB^{\alpha}$ (V_{max} = 0.19 ± 0.01 min^{-1} Lon_6^{-1}, K_M = 16 ± 2.4 μM). For comparison, the plots for IbpA and IbpB are shown in gray.

these experiments indicate that the tails of IbpA inhibit Lon degradation, whereas those of IbpB promote degradation.

To investigate the contribution of the Ibp tails to Lon recognition, we determined the concentration-dependence of $IbpA^{\alpha}$ and $IbpB^{\alpha}$ degradation (Fig. 2.2B, C). K_M values for

Lon degradation of IbpA$^{\alpha}$ and IbpB$^{\alpha}$ were nearly identical and were within error of those measured for IbpA and IbpB (Table 2.1). Once again, differences in the rates of Lon degradation were completely attributable to differences in V_{max} values (Fig. 2.2B, C; Table 2.1). These experiments demonstrate that the N- and C-terminal tails of the Ibps are dispensable for Lon recognition. Thus, unlike other specific substrates of Lon, the Ibps are recognized via sequence or structural determinants in the body of the protein, specifically, within the α-crystallin domains of the Ibps.

Lon degrades human α-crystallin domains.

Our results with the tailless Ibps clearly indicate that Lon recognizes a region within the α-crystallin domains of the Ibps. We hypothesized that Lon recognizes a feature of the natively folded α-crystallin domain and might therefore degrade other α-crystallin proteins. To test this model, we purified full-length versions of two human α-crystallin proteins, αA-crystallin (hαA) and αB-crystallin (hαB), as well as the tail-less α-crystallin domains of these proteins (hαA$^{\alpha}$ and hαB$^{\alpha}$), and assayed degradation by Lon *in vitro* (Fig. 2.3B,C and Table 2.1). Strikingly, hαA$^{\alpha}$ and hαB$^{\alpha}$ were both excellent Lon substrates. Indeed, the second-order rate constants (V_{max}/K_M) for Lon degradation of these α-crystallin domains were 2-fold to 4-fold larger than the value for IbpB, which was the best Lon substrate among the full-length and tail-less Ibp proteins. Furthermore, although the V_{max} values for Lon degradation of human αA, αB, αA$^{\alpha}$, and αB$^{\alpha}$ differed substantially (0.06-3.8 min^{-1} Lon$_6^{-1}$; Table 2.1), the K_M values were similar (26-58 μM). These experiments establish that Lon can degrade α-crystallin domains

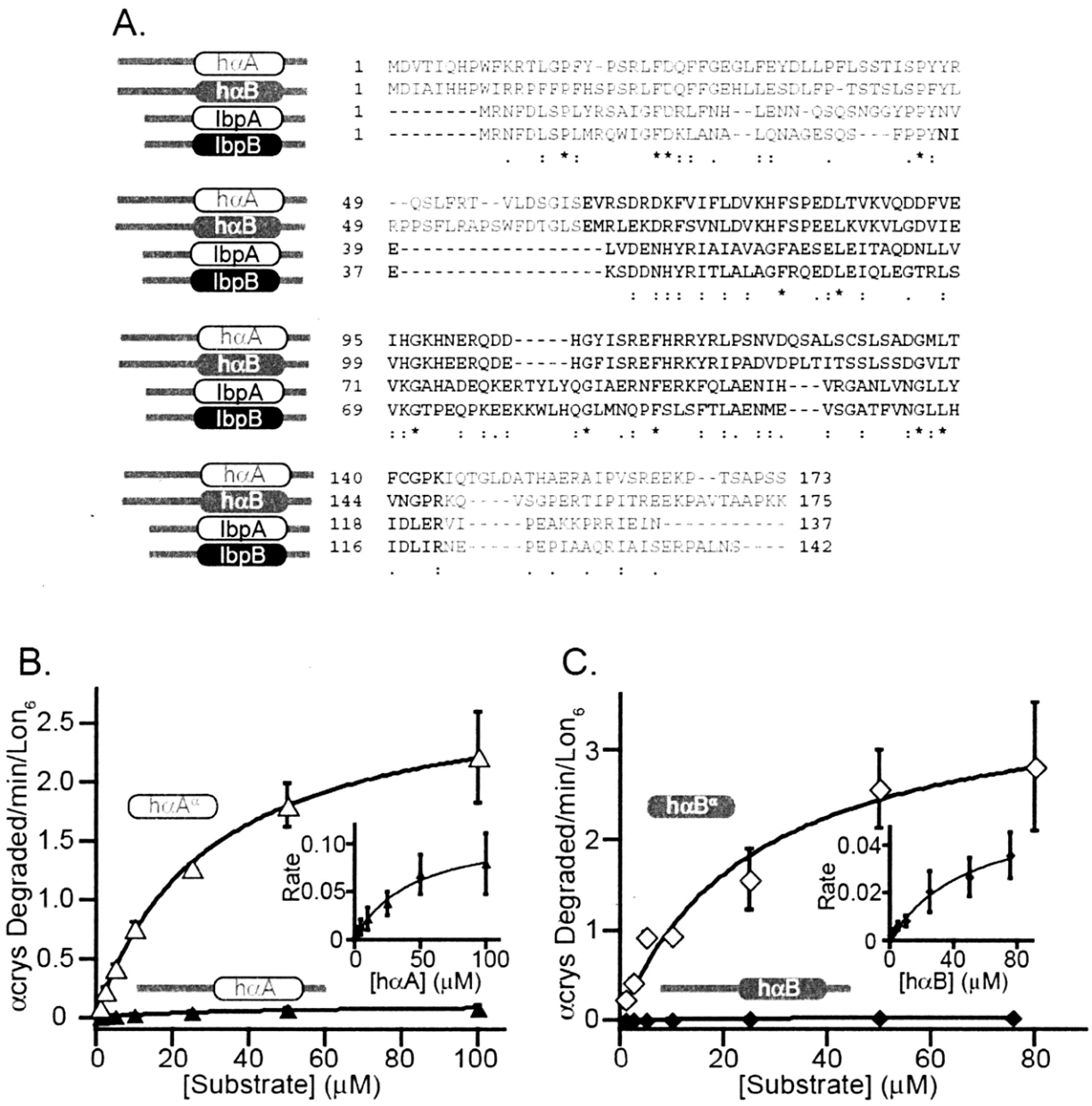

Figure 2.3: Lon degrades human α-crystallin proteins.
(A) Sequence alignment of *E. coli* IbpA and IbpB and human αA-crystallin and αB-crystallin generated by ClustalW2. (B) Michaelis-Menten plots for Lon degradation of full-length αA-crystallin (V_{max} = 0.12 ± 0.05 min^{-1} Lon$_6^{-1}$, K_M = 50 ± 20 μM) or αA$^{\alpha}$ (V_{max} = 2.5 ± 0.3 min^{-1} Lon$_6^{-1}$, K_M = 35 ± 8.9 μM). (C) Michaelis-Menten plots for Lon degradation of full-length αB-crystallin (V_{max} = 0.06 ± 0.02 min^{-1} Lon$_6^{-1}$, K_M = 58 ± 21 μM) or αB$^{\alpha}$ (V_{max} = 3.8 ± 1.4 min^{-1} Lon$_6^{-1}$, K_M = 26 ± 12 μM). Insets: The data for hαA and hαB are replotted on expanded scales.

that are only distantly related to the Ibps (Fig. 2.3A) and support our hypothesis that Lon may recognize a feature of the folded α-crystallin domain.

It is possible that the native and denatured forms of the α-crystallin domain are in dynamic equilibrium and that Lon recognizes a peptide sequence in the unfolded protein. However, given that all of these variants contain the same modular fold, but different amino-acid sequences, and were all degraded with similar K_M values, we consider it more likely that Lon recognizes a general characteristic of the folded α-crystallin domain.

The oligomeric-states of full-length and tail-less α-crystallin proteins do not correlate with their rates of degradation

The ability to form higher-order oligomers is thought to be of crucial importance for sHSPs to perform their chaperone functions (Fernando and Heikkila, 2000). Furthermore, oligomerization is disrupted by deletions in the N- and C-terminal tails of sHSPs (Fernando and Heikkila, 2000; Jiao *et al.*, 2005b). To investigate the possible relationship between the oligomeric state of the Ibps/human α-crystallin proteins and their rates of degradation by Lon, we performed gel filtration analysis on full length and tail-less IbpB (Fig. 2.4A) and full length and tail-less human αB-crystallin (Fig. 2.4B). Consistent with previous reports, we found that tail-less versions of both IbpB and αB-crystallin eluted as much smaller complexes than the full-length proteins. However, while $IbpB^{\alpha}$ is only degraded a third as fast as full length IbpB, αB^{α} is degraded over 60 times faster than full-length αB-crystallin (Fig. 2.2C, 2.3C, and Table 2.1). Thus, we find

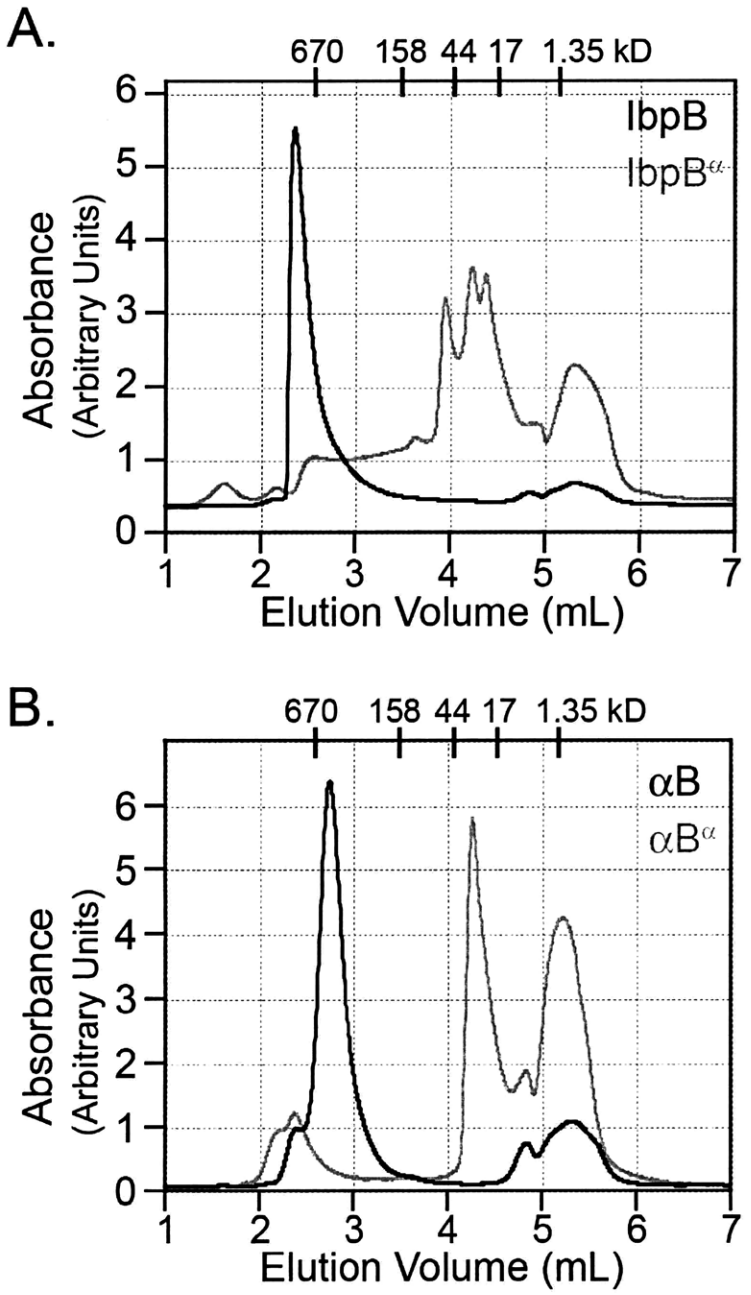

Figure 2.4: Tail-less *E. coli* IbpB and human αB-crystallin both elute as smaller oligomers than their full-length counterparts.
(A) Profile from the gel filtration chromatography of purified *E. coli* IbpB (black line) and IbpB$^{\alpha}$ (gray line) or (B) human αB-crystallin (black line) and αB$^{\alpha}$ (gray line). Protein was detected by measuring the absorbance at 280 nm or 213 nm, and is displayed in arbitrary units. The calculated monomer molecular weights of IbpB, IbpB$^{\alpha}$, αB, and αB$^{\alpha}$ are 16 kDa, 10 kDa, 20 kDa, and 10 kDa respectively. Tick marks at the top of the graph indicate where molecular weight standards eluted (more information about the standards is in the materials and methods section).

that Lon is able to degrade both highly oligomerized versions of α-crystallin proteins (IbpB), as well as smaller oligomeric versions of α-crystallin proteins (αB^{α}) in a robust manner. Therefore, the difference in multimeric state does not explain how the tails of the Ibps/α-crystallin proteins control their rate of degradation.

IbpB facilitates Lon degradation of IbpA *in vivo* and *in vitro*.

IbpA and IbpB interact and cooperate to perform chaperone functions (see introduction and Matuszewska *et al.*, 2005). To investigate whether either Ibp protein affects Lon degradation of the other molecule, we assayed Lon degradation of ^{35}S-IbpA in the presence of unlabeled IbpB and *vice versa*. Importantly, the rate of IbpA degradation increased seven-fold in the presence of equimolar IbpB, indicating that IbpB facilitates Lon degradation of IbpA. In contrast, the rate of IbpB degradation decreased two-fold in the presence of equimolar IbpA (Fig. 2.5A).

To determine if IbpB alters Lon degradation of IbpA *in vivo*, we measured the intracellular turnover of IbpA in the presence or absence of IbpB at 45 °C, where both

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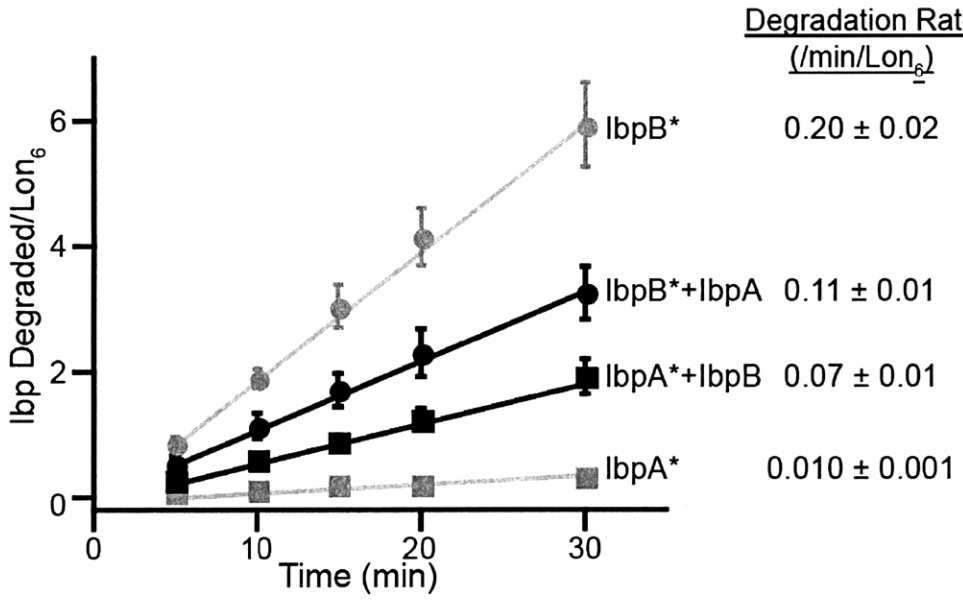
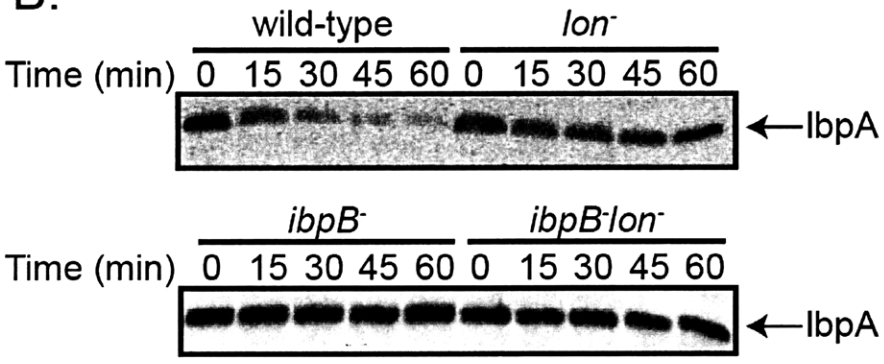
Figure 2.5: IbpB facilitates IbpA degradation both *in vitro* and *in vivo*.
(A) Degradation of 5 μM ^{35}S-IbpA (gray squares), 5 μM ^{35}S-IbpB (gray circles), 5 μM ^{35}S-IbpA with 5 μM unlabeled IbpB (black squares), and 5 μM ^{35}S-labeled IbpB with 5 μM unlabeled IbpA (black circles) by 600 nM Lon_6. Asterisks denote ^{35}S-labeled protein. (B) Western blots probed with an IbpA-specific antibody showing the time course of IbpA degradation in wild-type (top panel, left side), *lon*$^-$ (top panel, right side), *ibpB*$^-$ (bottom panel, left side), or *ibpB*$^-$*lon*$^-$ (bottom panel, right side) strains after inhibiting translation with spectinomycin. Representative blots from one of three independent experiments are shown. (C) Bands from the experiments in panel B were quantified, and relative intensities are plotted.

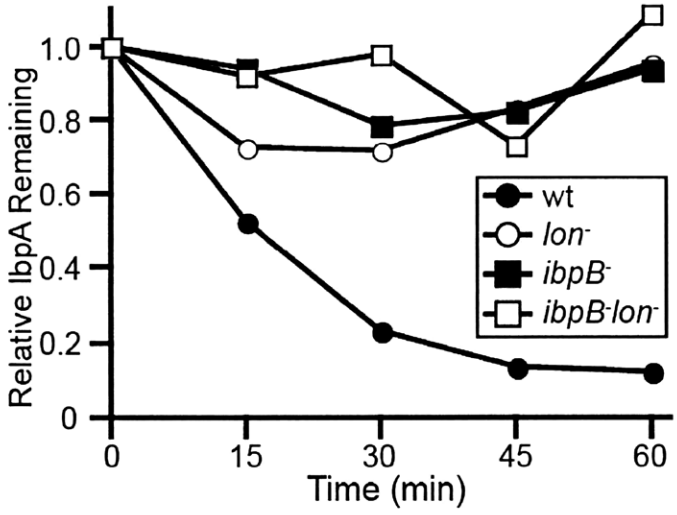
A.


Degradation Rate
(/min/Lon6)
IbpB*
0.20 ± 0.02
IbpB*+IbpA
0.11 ± 0.01
IbpA*+IbpB
0.07 ± 0.01
IbpA*
0.010 ± 0.001
Ibp Degraded/Lon6
Time (min)
0
2
4
6
0
10
20
30

B.


wild-type
lon-
Time (min) 0 15 30 45 60 0 15 30 45 60
IbpA
ibpB-
ibpB-lon-
Time (min) 0 15 30 45 60 0 15 30 45 60
IbpA

C.


Relative IbpA Remaining
Time (min)
0
0.2
0.4
0.6
0.8
1.0
0
15
30
45
60
wt
lon-
ibpB-
ibpB-lon-

proteins are expressed at reasonably high levels (Laskowska *et al.*, 1996). IbpA was degraded with a half-life of about 20 minutes in a *lon*$^+$ strain but was completely stabilized in an otherwise isogenic *lon*$^-$ strain (Fig. 2.5B, top panel and 2.5C), supporting the idea that under these conditions, Lon is the predominant protease responsible for IbpA degradation *in vivo*. Furthermore, in complete agreement with our *in vitro* experiment (Fig. 2.5A), IbpA was also stabilized in an *ibpB*$^-$ *lon*$^+$ strain (Fig. 2.5B, bottom panel and 2.5C), indicating that both IbpB and Lon are essential for efficient intracellular degradation of IbpA. Because our IbpB antibody was not as sensitive as the IbpA antibody, we were unable to detect endogenous levels of IbpB, even after heat-shock. However, in a strain lacking chromosomal *ibpAB*, over-expressed IbpB was degraded in a largely Lon-dependent manner (data not shown), consistent with the fact that Lon degrades IbpB in the absence of IbpA *in vitro*. These degradation results *in vivo* are also consistent with the finding that IbpA and IbpB were both recovered in substrate-trapping experiments with proteolytically inactive Lon (E. Oakes and TAB personal communication). Thus, we conclude that both IbpA and IbpB are Lon substrates and that IbpB facilitates Lon degradation of IbpA *in vitro* and *in vivo*, suggesting that some type of IbpA•IbpB•Lon interaction is important for properly controlled degradation.

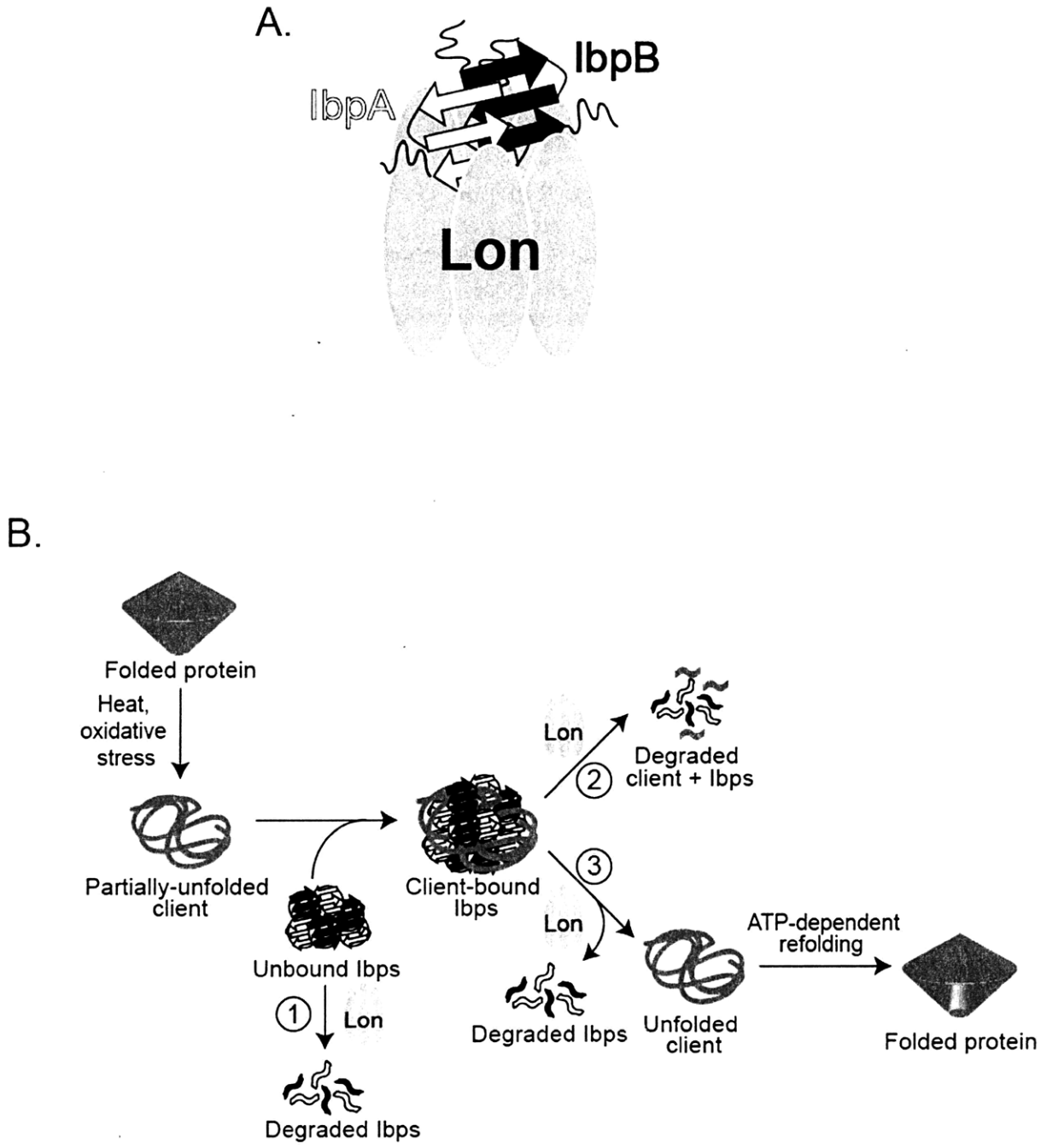


Figure 2.6: Models for Lon recognition and degradation of the Ibps.
(A) Lon recognizes the α-crystallin domains of IbpA and IbpB and not the N- and C-terminal tails of these proteins. (B) Model for the significance of Ibp degradation by Lon *in vivo*. We propose that Lon degrades free Ibps (1) as well as client-bound Ibps (2 and 3). (See text for details.)

Discussion

In this work, we demonstrate that Lon degrades the *E. coli* sHSPs, IbpA and IbpB. Unlike other specific Lon substrates (non-damaged proteins) whose recognition determinants are at the far N- or C-terminus of the substrate, the Lon-recognition determinants of the Ibps are located within the core α-crystallin domains of the Ibps. However, the N- and/or C-terminal tails of the Ibps play a critical role in controlling their rate of degradation. Interestingly, this control is manifested as a difference in the maximum rate of Ibp degradation and not as a difference in K_M. Finally we show that IbpA is degraded more efficiently by Lon when IbpB is present both *in vitro* and *in vivo*.

Lon recognition of Ibps.

AAA+ proteases typically recognize peptide signals near the N- or C-termini of substrates (Flynn *et al.*, 2003; Gottesman *et al.*, 1998; Tobias *et al.*, 1991), and Lon can also choose substrates by interacting with specific peptide signals (Choy *et al.*, 2007; Gonzalez *et al.*, 1998; Gur and Sauer, 2008a, 2008b; Ishii and Amano, 2001; Shah and Wolf, 2006a). However, neither IbpA nor IbpB contain an obvious sequence that resembles well-characterized Lon degradation tags. Instead, we propose that Lon recognizes a portion of the folded α-crystallin domains of IbpA and IbpB (Fig. 2.6A). This model explains our findings that Lon degrades the full-length Ibps, their tail-less α-crystallin domains, as well as human αA-crystallin and αB-crystallin and their tail-less counterparts. Furthermore, in each case, the K_M values for these degradation reactions ranged from 15-60 μM (Table 2.1), suggesting that Lon recognizes these variants with similar affinities. An alternative model is that Lon recognizes a common peptide motif of

these α-crystallin domains. However, this mechanism is unlikely given the lack of similarity between the amino acid sequences of these proteins (Fig. 2.3A). Moreover, human mitochondrial Lon degrades the folded form of the α-subunit of mitochondrial processing peptidase more efficiently than it degrades the unfolded form of this substrate (Ondrovicová *et al.*, 2005), providing a precedent for Lon recognition of a structural feature of a folded domain.

The tails of IbpA and IbpB control the rate of Lon degradation.

Interestingly, the V_{max} values for Lon degradation of different variants of IbpA, IbpB, αA-crystallin, and αB-crystallin varied substantially, in some cases by more than 50-fold (Table 2.1). Moreover, the tails of IbpA, αA-crystallin, and αB-crystallin slow degradation, whereas the tails of IbpB facilitate degradation. And although the tails of sHSPs are known to affect sHSP oligomerization, we found no correlation between the oligomeric state of Ibp/α-crystallin and their rate of degradation (Fig. 2.2C, 2.3C and 2.4). This ability of the protein tails to alter the maximal rate of degradation is unusual, as degradation by AAA+ proteases is usually regulated at the level of recognition (for review, see Baker and Sauer, 2006). However, recent studies show that model substrates can program the speed and efficiency of degradation by Lon (Gur and Sauer, 2009). The Ibps therefore appear to be examples of biological Lon substrates whose degradation can be controlled in this manner.

IbpB activates Lon degradation of IbpA.

Our studies show that IbpB facilitates Lon degradation of IbpA both *in vitro* and *in vivo* (Fig. 2.5). This apparent interaction parallels the ability of IbpA and IbpB to cooperate in performing their chaperone activity *in vitro* (Matuszewska *et al.*, 2005; Ratajczak *et al.*, 2009). However, to our knowledge, cooperation of any kind between endogenous IbpA and IbpB *in vivo* has not previously been described. It remains to be determined whether IbpB-controlled degradation of IbpA by Lon serves a regulatory role or is tied, in some fashion, to chaperone activities *in vivo*.

Nevertheless, these connections between the assembled Ibps and Lon suggest that there may be functional cross-talk between the sHSPs and the protein-degradation machinery. For example, IbpA and IbpB may be degraded by Lon as they cooperate to help Lon recognize and degrade damaged proteins, which cannot be refolded (Fig. 2.6B). In this model, IbpA and IbpB act analogously to the *Bacillus subtilis* MecA adaptor protein, which facilitates the degradation of unfolded and aggregated substrates by the protease ClpCP *in vitro* and is itself degraded in the process (Schlothauer *et al.*, 2003; Turgay *et al.*, 1998). We propose that Lon also degrades free Ibps in order to keep their concentrations low during non-stress conditions. In this case, Lon degradation of unneeded Ibps might serve to prevent spurious Ibp binding to functional proteins.

The model shown in Fig. 2.6B is supported by several lines of evidence. In the absence of client proteins, we find that the Ibps are degraded by Lon *in vitro* (Fig. 2.1B and C), making it likely that degradation of unbound Ibps occurs *in vivo* as well (Fig. 2.6B(1)).

Furthermore, under heat stress *in vivo*, when the Ibps would be expected to be bound to client proteins (Laskowska *et al.*, 1996), we find that IbpA is degraded robustly by Lon (Fig. 2.5B and C). We propose two possibilities for why Lon might degrade client-bound Ibps *in vivo*. One possibility is that, as described above, the Ibps are delivering bound client proteins to Lon, and are being degraded in the process (Fig 2.6B(2)). Alternatively, the role of degradation of client-bound Ibps might be to remove Ibps from client proteins, thereby facilitating client protein refolding (Fig. 2.6B (3)). It has been demonstrated *in vitro* that ClpB greatly accelerates DnaKJE-mediated refolding of sHSP-bound aggregated proteins, suggesting that ClpB is able to disaggregate sHSP-client complexes (Mogk *et al.*, 2003b; Ratajczak *et al.*, 2009). But given our data, which suggests that Lon can degrade client-bound Ibps *in vivo* (Fig. 2.5B), it is tempting to speculate that Lon may also contribute to the removal of the Ibps from client proteins.

Finally, our data clearly indicate that IbpB is required for the efficient degradation of IbpA (Fig. 2.5). Because IbpA and IbpB are co-transcribed, IbpA is expected to be translated first. As a result, a mechanism could have evolved to prevent Lon degradation of IbpA before it binds to its partner, IbpB. By this reasoning, IbpA degradation is unlikely to simply be a way to clear unassembled subunits but rather is likely to serve an important functional role. Further experiments will be needed to interrogate models for the biological roles of the Ibp-Lon collaboration.

Materials and Methods

Protein Purification:

All Ibp and human α-crystallin proteins were purified using a method modified from Malakhov *et al.* The human α-crystallin constructs were a gift from Ligia Acosta-Sampson and Jonathan King, MIT. His-SUMO fusions of each gene were cloned downstream of the T7 promoter and expressed in BL21 *Δibp::kan*. Cultures of each strain (1 L) were grown in LB broth at 37 °C, induced at OD_{600} 0.8 with 0.5 mM IPTG, grown for an additional three hours at 30 °C, harvested, and stored frozen at -80 °C until purification. Cells were thawed, resuspended in 3 mL of 50 mM HEPES-KOH (pH 8.0), and 0.5 mL of the nuclease Benzonase (Novagen) was added. The cells were lysed in a cell disruptor (Constant Systems) at 25 MPa, 10 mL of lysate dilution buffer (50 mM HEPES-KOH [pH 8.0], 4.5 M urea, 1.5 mM β-mercaptoethanol [BME], 600 mM potassium glutamate) was added, the lysate was cleared by centrifugation at 30000 x g for 20 minutes, and the supernatant was incubated with 2 mL Ni-NTA beads (Qiagen) for 30 min at 4 °C. The beads were packed in a column, rinsed with 250 mL LG-5 buffer (50 mM HEPES-KOH [pH 8.0], 4 M urea, 1 mM BME, 400 mM potassium glutamate, 5 mM imidazole) followed by 250 mL LG-20 buffer (identical to LG-5 buffer except with 20 mM imidazole). The protein was eluted with 10 mL elution buffer (50 mM HEPES-KOH [pH 8.0], 3 M urea, 1 mM BME, 400 mM potassium glutamate, 500 mM imidazole, 10% glycerol). Peak fractions were pooled and dialyzed overnight against 2 L of dialysis buffer 1 (50 mM HEPES-KOH [pH 8.0], 2 M urea, 10% glycerol, 200 mM potassium glutamate, 1 mM BME). The His-SUMO domain was cleaved off by incubating the dialyzed protein with 100 μL of ULP protease (purified as in Malakov *et al.*) for six hours

at 37 °C. The protein was then flowed over 2 mL packed Ni-NTA beads, and the flow-through fraction was collected and dialyzed against 2 L dialysis buffer 2 (50 mM HEPES-KOH [pH 8.0], 800 mM potassium glutamate, 20% sucrose, 1 mM BME). The molecular weight of each protein was confirmed by MALDI mass spectrometry.

^{35}S-labeled versions of substrates were expressed and purified as follows. Cultures of 100 mL were grown in LB broth at 37 °C to an OD_{600} of 1.0. Cells were harvested and resuspended in 100 mL of M9 media supplemented with MAM and grown for an additional 15 min at 37 °C. Cultures were induced with 0.5 mM IPTG and grown for an additional 20 min. ^{35}S-methionine (3.5 mCi) was added, the culture was grown for 3.5 hours at 37 °C, and the cells were harvested and subsequently resuspended in 3 mL of 50 mM HEPES-KOH (pH 8.0). Lysozyme was then added to a final concentration of 0.3 mg/ml, the cells were incubated on ice for 20 min, and then frozen at -80 °C until purification. ^{35}S-labelled proteins were purified essentially as described above. Lon was purified using the procedure of Gur and Sauer (Gur and Sauer, 2008b).

The isolated α-crystallin domains of IbpA, IbpB, αA-crystallin, and αB-crystallin consisted of the following residues: IbpA (40-123), IbpB (36-121), αA-crystallin (63-145), αB-crystallin (67-149). A methionine was added to the N-terminus of each of these constructs to facilitate labeling with ^{35}S-methionine.

Degradation *in vitro*:

Degradation of substrates *in vitro* was performed using 600 nM Lon_6 in 50 mM Tris (pH 8.0), 15 mM $MgCl_2$, 5 mM KCl, 1 mM DTT, 2% DMSO, 25 mM HEPES-KOH (pH 8.0), 400 mM potassium glutamate, 10% sucrose, and 0.5 mM BME. Lon and substrate were incubated at 37 °C for 2 min before the addition of the ATP-regeneration mix (32 mM ATP, 400 mg/ml creatine kinase, 40 mM creatine phosphate). Time points were taken every 30 sec for 2.5 min, or every 5 min for 30 min. Reactions were quenched in 10% trichloroacetic acid (TCA). The TCA-insoluble material was removed by centrifugation and ^{35}S radioactivity in the supernatant was measured in a Tri-Carb liquid scintillation counter (Perkin Elmer). The fraction of substrate that had been degraded at each time point was calculated by dividing the TCA-soluble counts by the total counts in an equal volume of each reaction.

Determination of oligomeric-state by gel filtration chromatography:

50 μL of IbpB, IbpB$^{\alpha}$, αB-crystallin, or αB$^{\alpha}$ (70 μM each) was loaded onto a Tosoh Super SW 3000 HPLC column (pre-equilibrated with a buffer containing 50 mM HEPES-KOH [pH 8.0] and 300 mM potassium glutamate) and run at 0.3 mL/min. Peaks were detected by measuring the absorbance at 280 nm or 213 nm. A mix of molecular weight standards (BioRad), which included thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa) and vitamin B_{12} (1.35 kDa), were run under the same conditions.

Antibody production:

Polyclonal antibodies specific to IbpA and IbpB were generated by inoculating rabbits with a peptide specific to IbpA (RVIPEAKKPRRIEIN) or IbpB (IDLIRNEPEPIAAQR). Antibodies were produced by Covance Research Products.

Degradation *in vivo*:

For degradation assays *in vivo*, a 60 mL culture in LB broth was grown at 37 °C in a shaking water bath to OD_{600} 0.2. The culture was then subjected to 45 °C heat-shock for 30 minutes before a 900 μL aliquot of the culture was added to 100 μL 100% TCA for the t=0 sample. Spectinomycin was then added (to 400 μg/ml), and samples were taken at 15 min intervals for 60 minutes. For each sample, OD_{600} was determined, and then TCA was added to a final concentration of 10%. The cultures were maintained at 45°C during the time-course. Samples were recovered by centrifugation and the pellets were rinsed with acetone and resuspended in enough 2X Tris-tricine sample buffer so that each sample contained the same cell density. An equal volume of each sample was run on a 16.5% Tris-tricine polyacrylamide gel. IbpA levels were detected using the anti-IbpA antibody described above and were quantified using ImageQuant software (GE Health Sciences).

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Chapter Three

IbpB facilitates IbpA degradation by directly activating IbpA, not by activating Lon

Izarys Rivera-Rivera contributed Figure 3.1 of this chapter.

Abstract

The *E. coli* small heat-shock proteins (sHSPs) IbpA and IbpB are substrates of the AAA+ protease Lon. Although the rate of degradation of IbpB is 15-fold faster than that of IbpA, in the presence of equimolar IbpB, the rate of IbpA degradation is accelerated 7-fold. Here we investigate the mechanism by which IbpB facilitates IbpA degradation. Like all sHSPs, IbpA and IbpB contain a conserved, central α-crystallin domain, flanked by N- and C-terminal tails. We find that the N- and C-terminal tails of IbpB are essential for IbpB to stimulate IbpA degradation. Furthermore, we find that although higher-order oligomerization of IbpB may be necessary for IbpB-facilitated IbpA degradation, it is not sufficient. We considered two general models to explain IbpB-stimulated degradation of IbpA. The first is that IbpB, through its tails, is acting as an allosteric activator of Lon, thereby leading to an increase in IbpA degradation. The second model is that IbpB, through its tails, is acting directly and specifically on IbpA to make IbpA a better substrate. We were unable to find any evidence that IbpB allosterically activates Lon. In contrast, we find that IbpB specifically stimulates IbpA degradation, and that IbpA degradation is only stimulated by IbpB. Our results indicate that the most likely explanation for IbpB-facilitated IbpA degradation is that IbpB acts directly and specifically on IbpA to make IbpA a better substrate, thus stimulating IbpA degradation.

Introduction

Small heat-shock proteins (sHSPs) are molecular chaperones that bind misfolded proteins, protecting them from irreversible aggregation until they can be refolded by ATP-dependent refoldases (Lee *et al.*, 1997; Mchaourab *et al.*, 2009). As such, small heat-shock proteins make up one arm of the protein quality-control network, a network that also includes disaggregases, refoldases, and proteases (Narberhaus, 2002).

All sHSPs consist of a conserved, central α-crystallin domain flanked by N- and C-terminal tails of varying lengths and sequence composition (Haslbeck *et al.*, 2005). Although poorly conserved, the N- and C-terminal tails are essential for higher-order oligomer formation, a feature of the sHSPs that is in turn thought to be critical for their chaperone function (Bagnéris *et al.*, 2009; Fernando and Heikkila, 2000).

The *E. coli* genome encodes two sHSPs, IbpA and IbpB. The amino acid sequences of IbpA and IbpB are approximately 50% identical and they are transcribed from the same operon (Allen *et al.*, 1992). The Ibps are involved in protecting *E. coli* from heat and oxidative stress, as well as copper-induced stress (Kitagawa *et al.*, 2000; Matuszewska *et al.*, 2008). Recently the Ibps have also been implicated in biofilm formation (Kuczynska-Wisnik *et al.*, 2009). Mechanistically, the Ibps are thought to bind unfolded proteins and to cooperate with the ClpB disaggregase as well as the DnaK/DnaJ/GrpE (DnaKJE) system, allowing for the refolding of aggregated proteins *in vivo* (Mogk *et al.*, 2003a). And in fact *in vitro*, the Ibps cooperate with ClpB, the DnaKJE system, and GroEL/ES chaperones to refold different aggregated model substrates (Ratajczak *et al.*,

2009; Veinger *et al.*, 1998). Intriguingly, despite the fact that their amino acid sequences show close to 50% sequence identity, IbpA and IbpB seem to have distinct activities. Specifically, IbpA but not IbpB is competent to keep the model client protein luciferase in morphologically smaller aggregates, while IbpB is required for the refolding of luciferase by the ClpB/DnaKJE systems (Ratajczak *et al.*, 2009).

Consistent with this idea that IbpA and IbpB have distinct activities, we recently demonstrated that IbpA and IbpB are degraded at different rates by Lon, a protease in the AAA+ (ATPases associated with various cellular activities) family of enzymes (Chapter 2, Fig. 2.1). Specifically, we found that the maximal rate of degradation of IbpB is 15-fold faster than that of IbpA (Chapter 2, Fig. 2.1). Intriguingly, we found that IbpB stimulates IbpA degradation both *in vitro* and *in vivo* (Chapter 2, Fig 2.5), indicating that IbpA and IbpB cooperate in their degradation by Lon.

Here we investigate the mechanistic basis of IbpB-stimulated degradation of IbpA. We demonstrate that the N- and/or C-terminal tails of IbpB are critical for IbpB to facilitate IbpA degradation. Furthermore, higher-order oligomerization of IbpB may be required, but is not sufficient for IbpB-facilitated IbpA degradation. Two general models for how IbpB may be helping in the degradation of IbpA are proposed. The first model is that IbpB is a general allosteric activator of Lon, and that this activation of Lon leads to faster IbpA degradation. The second model is that IbpB acts directly on IbpA, making IbpA a better Lon substrate, which leads to faster IbpA degradation. Our results argue that

IbpB acts directly and specifically on IbpA, rather than acting as a general allosteric activator of Lon.

Results

IbpB, but not IbpA$^{\alpha}$ or IbpB$^{\alpha}$ facilitates the degradation of IbpA by Lon

We previously demonstrated that IbpB facilitates the degradation of IbpA both *in vitro* and *in vivo* (Chapter 2, Fig. 2.5). Therefore, we decided to investigate the biochemical mechanism by which IbpB facilitates IbpA degradation. Given that the tails of the Ibps are not required for the recognition of the Ibps by Lon (Chapter 2, Fig. 2.2), we asked if tail-less IbpA and IbpB (referred to as IbpA$^{\alpha}$ and IbpB$^{\alpha}$ hereafter), which are both substrates of Lon and are degraded faster than IbpA (Chapter 2, Fig. 2.2), would be able to facilitate the degradation of IbpA. To answer this question, we added unlabeled IbpB, IbpA$^{\alpha}$ or IbpB$^{\alpha}$ to a degradation reaction containing Lon and ^{35}S-labeled IbpA, and measured the degradation of IbpA by monitoring the appearance of TCA-soluble ^{35}S-labeled peptides over time. As expected, IbpB stimulated the degradation of IbpA

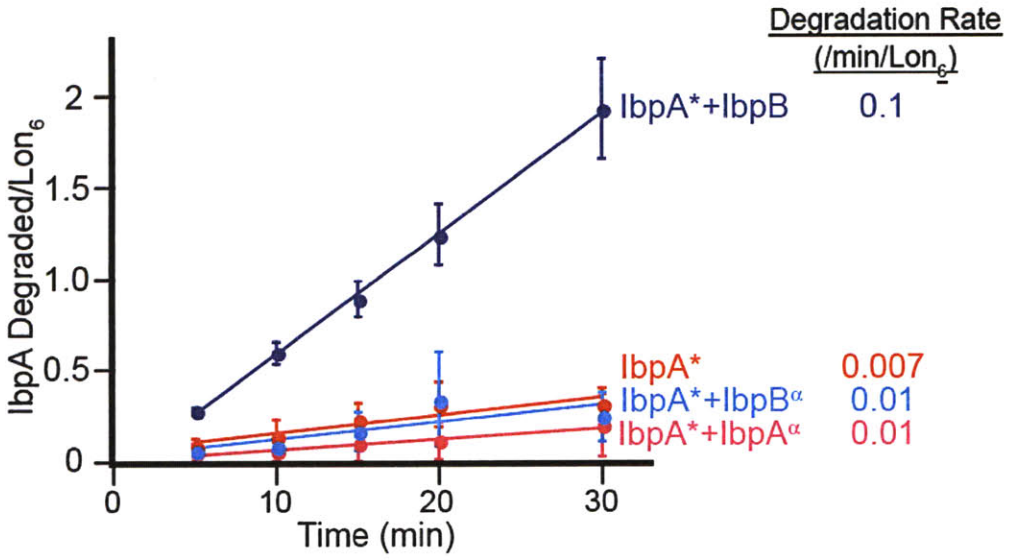

Figure 3.1: The tails of IbpB are necessary for IbpB to facilitate IbpA degradation
Rate of degradation of 5 μM ^{35}S-labeled IbpA alone (red) or in the presence of 5 μM unlabeled IbpB (dark blue), IbpB$^{\alpha}$ (light blue), or IbpA$^{\alpha}$ (pink), by 600 nM Lon_6.

(Fig. 3.1, dark blue line). However, neither IbpA$^{\alpha}$ nor IbpB$^{\alpha}$ stimulated the degradation of IbpA (Fig. 3.1, pink and light blue lines respectively). These data indicate that the N- and/or C-terminal tails of IbpB are required for IbpB to stimulate IbpA degradation, as IbpB$^{\alpha}$ was unable to facilitate IbpA degradation.

Characterization of chimeric IbpB^{AT}

The tails of sHSPs in general, and of IbpB in particular, are known to be involved in higher-order sHSP oligomerization (Bagnéris *et al.*, 2009; Jiao *et al.*, 2005b). Tail-less IbpB forms much smaller oligomers (2-4mers), compared to full-length IbpB (45-mers+) (Chapter 2, Fig. 2.4). Therefore, we were interested in whether the inability of IbpB$^{\alpha}$ to facilitate IbpA degradation was due, not to the absence of the IbpB tails *per se*, but due to the inability of IbpB$^{\alpha}$ to form higher-order oligomers. To differentiate between the hypothesis that IbpB oligomerization facilitates IbpA degradation, and the hypothesis that a separate feature of the IbpB tails allows IbpB to facilitate IbpA degradation, we needed a mutant version of IbpB that still oligomerized, but did not have the IbpB tails. In an attempt to make such a mutant, we constructed a chimeric version of IbpB composed of the α-crystallin domain of IbpB, flanked by the N- and C-terminal tails of IbpA. This protein will be referred to as IbpB^{AT} (for IbpB with IbpA Tails).

To determine the oligomeric state of IbpB^{AT}, we performed gel filtration analysis of IbpB^{AT} and compared it to the gel filtration profile of wild-type full-length IbpB. Like full-length IbpB (Fig. 3.2A), IbpB^{AT} also formed very large oligomers (Fig. 3.2B). The major IbpB^{AT} peak migrated as a complex larger than the 670 kDa standard, suggesting that

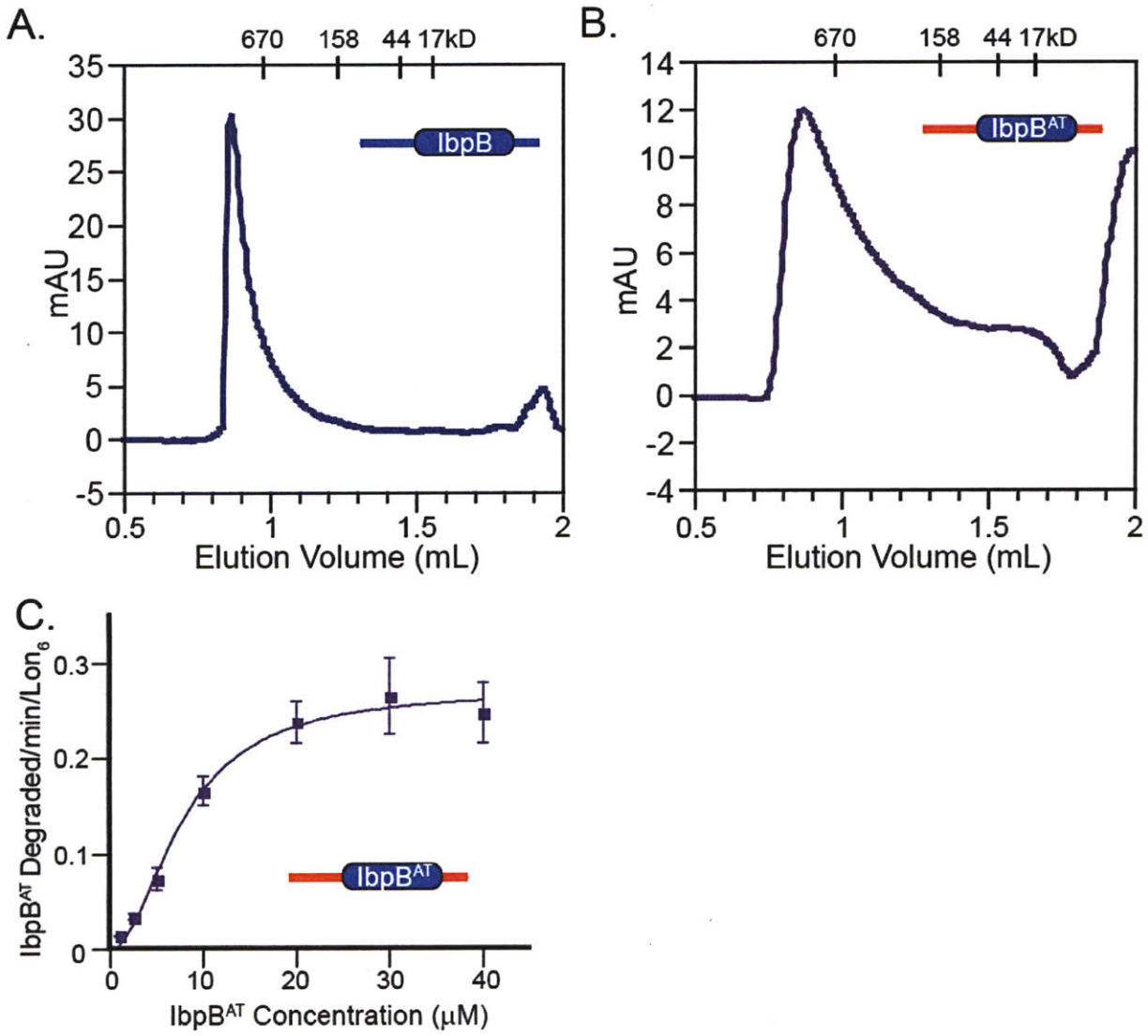

Figure 3.2: IbpB^{AT} forms higher-order oligomers and is a Lon substrate.
(A) Profile from the gel filtration chromatography of IbpB. (B) Profile from the gel filtration chromatography of IbpB^{AT}. The absorbance scale on this profile is different from that of (A) because IbpB^{AT} does not absorb as well at 280 nm because there is a tryptophan in the IbpB N-terminal tail that is not in the IbpA N-terminal tail. (C) Concentration-dependence of the degradation rate of IbpB^{AT} by 600 nM Lon_6. The data were fit to the Hill-equation.

IbpB^{AT} oligomers are made up of over 40 subunits (Fig. 3.2B). However, the major peak of IbpB^{AT} was also broader than that of wild-type IbpB, suggesting a greater degree of heterogeneity in the size distribution of IbpB^{AT} oligomers. These data indicate that the

α-crystallin domain of IbpB does not require its own tails to form higher-order oligomers. Furthermore this result demonstrates that $IbpB^{AT}$ is competent to form higher-order oligomers.

To further characterize $IbpB^{AT}$, we measured the steady-state kinetic parameters of the degradation of $IbpB^{AT}$ by Lon. The rates of $IbpB^{AT}$ degradation at increasing substrate concentrations were fit to the Hill equation, as we observed cooperativity in the degradation of $IbpB^{AT}$ (Hill coefficient ~1.9) (Fig. 3.2C). This cooperativity can be attributed to the observation that at lower concentrations, $IbpB^{AT}$ does not oligomerize as well as it does at higher concentrations (data not shown). We found that $IbpB^{AT}$ was degraded by Lon with an apparent half-maximal concentration of 8 μM (Fig. 3.2C). This apparent half-maximal concentration is relatively close to the K_M values we observed for the full-length and tail-less versions of IbpA and IbpB (16-18 μM). This result is therefore consistent with our previous hypothesis, that Lon recognizes the Ibps not through their N- and C-terminal tails, but through their core α-crystallin domains.

Higher-order oligomerization may be necessary, but is not sufficient for IbpB to facilitate IbpA degradation.

Given that $IbpB^{AT}$ forms higher-order oligomers, despite lacking the tails of IbpB, we next tested whether $IbpB^{AT}$ could facilitate the degradation of IbpA. To do this we added different ratios of unlabeled IbpB or $IbpB^{AT}$ to degradation reactions containing ^{35}S-labeled IbpA and Lon. Interestingly, we found that there does not need to be a 1:1

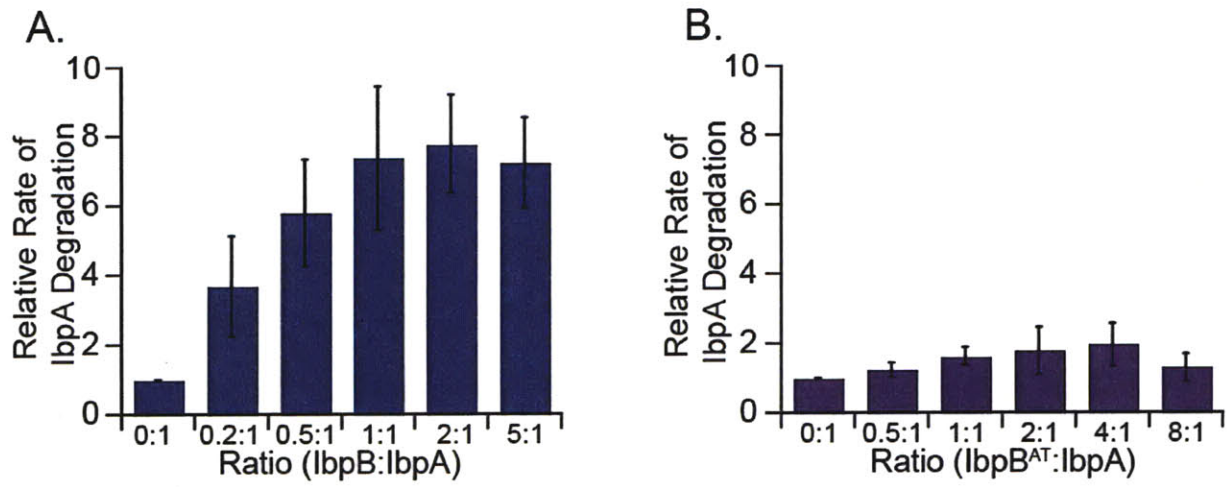

Figure 3.3: IbpB^{AT} does not stimulate IbpA degradation.
(A) Relative degradation rate of 5 μM ^{35}S-labeled IbpA in the absence (0:1) or presence of increasing amounts of unlabeled IbpB. (B) Relative degradation rate of 5 μM ^{35}S-labeled IbpA in the absence (0:1) or presence of increasing amounts of unlabeled IbpB^{AT}. For (A) and (B) the rates of degradation were normalized to the rate of degradation of IbpA alone.

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ratio of IbpB:IbpA for IbpB to facilitate IbpA degradation. This conclusion is based on the observation that the fold enhancement of IbpA degradation when the ratio of IbpB:IbpA is 0.5:1 (~6 fold enhancement) is very close to the enhancement observed with a 1:1 ratio (~7 fold enhancement) (Fig. 3.3A). Whereas if a 1:1 ratio of IbpB:IbpA were required, it is likely that the fold-enhancement observed with the 0.5:1 ratio of IbpB:IbpA would have been far less than the enhancement observed with the 1:1 ratio of IbpB:IbpA.

Interestingly, we found that IbpB^{AT} did a much poorer job of facilitating the degradation of IbpA than did IbpB. At equal molar concentrations of IbpB^{AT}:IbpA, we observed barely a 1.5-fold increase in the rate of IbpA degradation (Fig. 3.3B), compared to the 7-fold

increase in the rate of IbpA degradation when equimolar IbpB was added (Fig. 3.3A). Since $IbpB^{AT}$ is able to form higher-order oligomers (Fig. 3.2B), but is unable to robustly facilitate IbpA degradation (Fig. 3.3B), we conclude that higher-order oligomerization of IbpB may be necessary, but is not sufficient for IbpB to facilitate IbpA degradation. Therefore there must be a feature or features within the IbpB tails themselves that either on their own, or together with the α-crystallin domain of IbpB, enables IbpB to facilitate IbpA degradation.

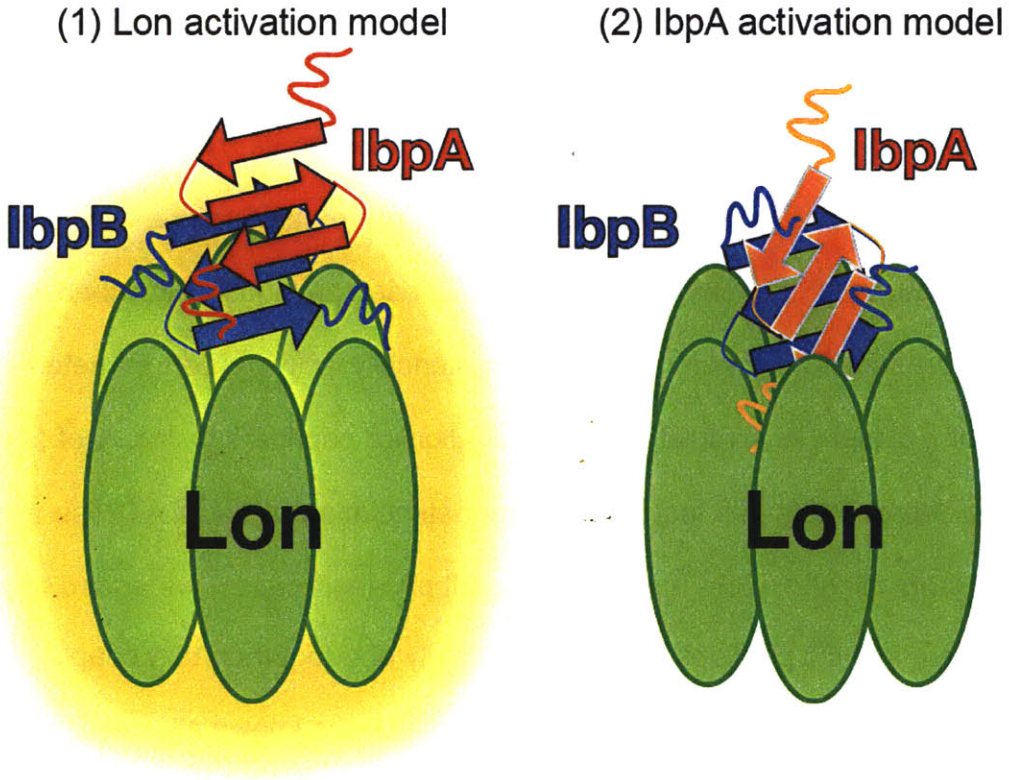

Figure 3.4: Two possible models for the activation of IbpA degradation by IbpB
The first model (1) is that IbpB through its tails is directly activating Lon, making Lon a better enzyme, which leads to the increased rate of IbpA degradation. The second model (2) is that IbpB through its tails is directly activating IbpA, presumably by eliciting a conformational change, which makes IbpA a better substrate, therefore leading to the increased IbpA degradation rate.

The IbpB tail peptides do not activate Lon

Given that the IbpB tails are critical for IbpB to facilitate IbpA degradation, we considered two simple models for how IbpB stimulates IbpA degradation. These models are as follows (Fig. 3.4). The first model is that IbpB, through its tails, is acting directly on Lon to allosterically activate and thus make Lon a better enzyme, leading to faster degradation of IbpA. We refer to this as the Lon activation model (Fig. 3.4(1)). The second model is that IbpB, through its tails, is acting directly on IbpA making IbpA a better substrate for Lon, leading to faster degradation of IbpA. We refer to this as the IbpA activation model (Fig. 3.4(2)).

If the Lon activation model is correct and IbpB, through its tails, is making Lon a better enzyme, we might expect that the tail peptides of IbpB alone would enhance the ATPase activity of Lon. This phenomenon has been previously described for α-casein, a model Lon substrate that is known to allosterically activate Lon as determined by the ability of α-casein to stimulate the degradation of peptide substrates of Lon (Waxman and Goldberg, 1986). To test the idea that stimulation of Lon's ATPase activity by the tails of IbpB might explain IbpB-stimulated IbpA degradation, we added the purified N- and C- terminal tail peptides of IbpB, separately and together, to a coupled Lon ATPase assay. As a comparison we confirmed that α-casein, a known allosteric activator of Lon, was able to enhance the ATPase rate of Lon more than four-fold (Fig. 3.5, black bar). Indeed, we observed a 3.5-fold increase in Lon ATPase activity in the presence of 50 μM each of both N- and C-terminal IbpB tail peptides (Fig. 3.5, blue bars). However, we saw an identical pattern of stimulation when we added the purified tail peptides of IbpA

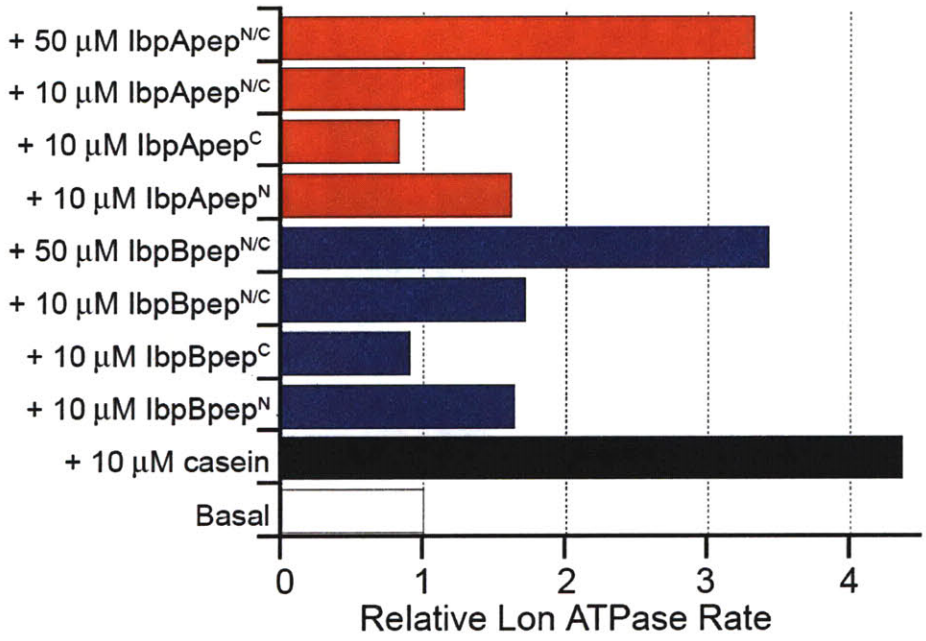

Figure 3.5: Activation of the ATPase rate of Lon by the IbpB tails cannot account for IbpB-stimulated IbpA degradation.
The relative ATPase rate of 150 nM Lon_6 in the presence of the indicated protein or peptides. The superscripted N or C refers to whether the N- or C-terminal tail peptide was added. N/C denotes that the indicated concentration of each of the N- and C-terminal tail peptides was added. ATPase rates were normalized to the basal ATPase rate of Lon alone.

(Fig. 3.5, red bars). These data demonstrate that an IbpB tail peptide-mediated enhancement of Lon ATPase activity can not explain the ability of IbpB to facilitate IbpA degradation, as there was no difference between the IbpB tail peptides and the IbpA tail peptides in their ability to accelerate the ATPase rate of Lon.

Despite the lack of difference between the ability of IbpA and IbpB tail peptides to enhance the Lon ATPase rate, this did not rule out the Lon activation model, as the ATPase rate of AAA+ proteases does not necessarily correlate with their ability to degrade substrate (Gur and Sauer, 2009; Hou *et al.*, 2008). Therefore, to further test the Lon activation model, we measured the rate of IbpA degradation in the presence of

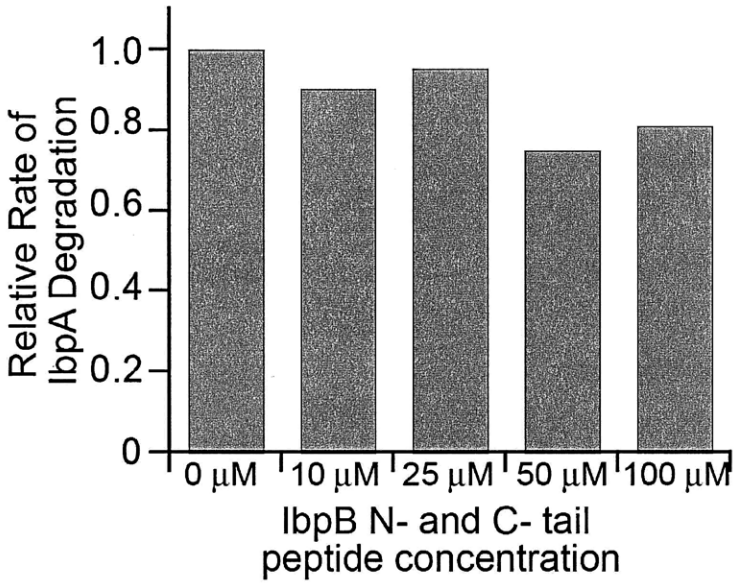

Figure 3.6: IbpB N- and C-terminal tail peptides alone are not sufficient to activate IbpA degradation.
The relative rate of degradation of 5 μM ^{35}S-labeled IbpA in the presence of increasing concentrations of the N- and C-terminal tail peptides of IbpB. Each reaction contained the indicated concentration of both the N- and C-terminal tail peptides. Degradation rates were normalized to the rate of degradation of 5 μM IbpA alone.

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increasing concentrations of IbpB tail peptides. Given that the N- and C-terminal tails of IbpB are necessary for IbpB-stimulated IbpA degradation, if the tails are responsible for activating Lon, we might expect that the tails alone would stimulate IbpA degradation. However, we saw no increase in IbpA degradation with the addition of increasing concentrations of IbpB tail peptides (Fig. 3.6). These results indicate that although the IbpB tails are necessary for IbpB to facilitate IbpA degradation, they are not on their own sufficient to facilitate IbpA degradation.

IbpB only stimulates IbpA degradation

Although the IbpB tail peptides alone were not able to accelerate the ATPase rate of Lon any better than the IbpA tail peptides (Fig. 3.5), nor did the IbpB tail peptides alone facilitate IbpA degradation (Fig. 3.6), these results still did not rule out the Lon activation model, since it is possible that the tails of IbpB need to work together with the α-crystallin domain of IbpB to activate Lon. Therefore, as a final test of the Lon activation model, we measured the ability of IbpB to facilitate the degradation of other Lon substrates. If IbpB facilitates IbpA degradation by allosterically activating Lon, this phenomenon should be a general one, and this activation of Lon should also be manifested in the faster degradation of other Lon substrates. This is seen in the case of a bona fide allosteric activator of Lon, α-casein, which facilitates the degradation of multiple Lon substrates (Waxman and Goldberg, 1986). On the other hand, if IbpA is the only substrate whose degradation is facilitated by IbpB, this specificity of activation would argue for the IbpA activation model.

To test these hypotheses, we measured the rate of degradation of α-casein in the presence or absence of IbpB. We did not detect any enhancement in the rate of α-casein degradation when IbpB was present in the reaction (Fig. 3.7A). To ensure that this lack of enhancement was not due to the already rapid degradation of α-casein, we also tested the degradation of human αA- and αB-crystallin, as well as $IbpA^{\alpha}$ and $IbpB^{\alpha}$, in the presence or absence of IbpB. The maximal rates of degradation of these proteins are all much slower than that of α-casein and range from 0.06-0.47 $min^{-1}Lon_6^{-1}$.

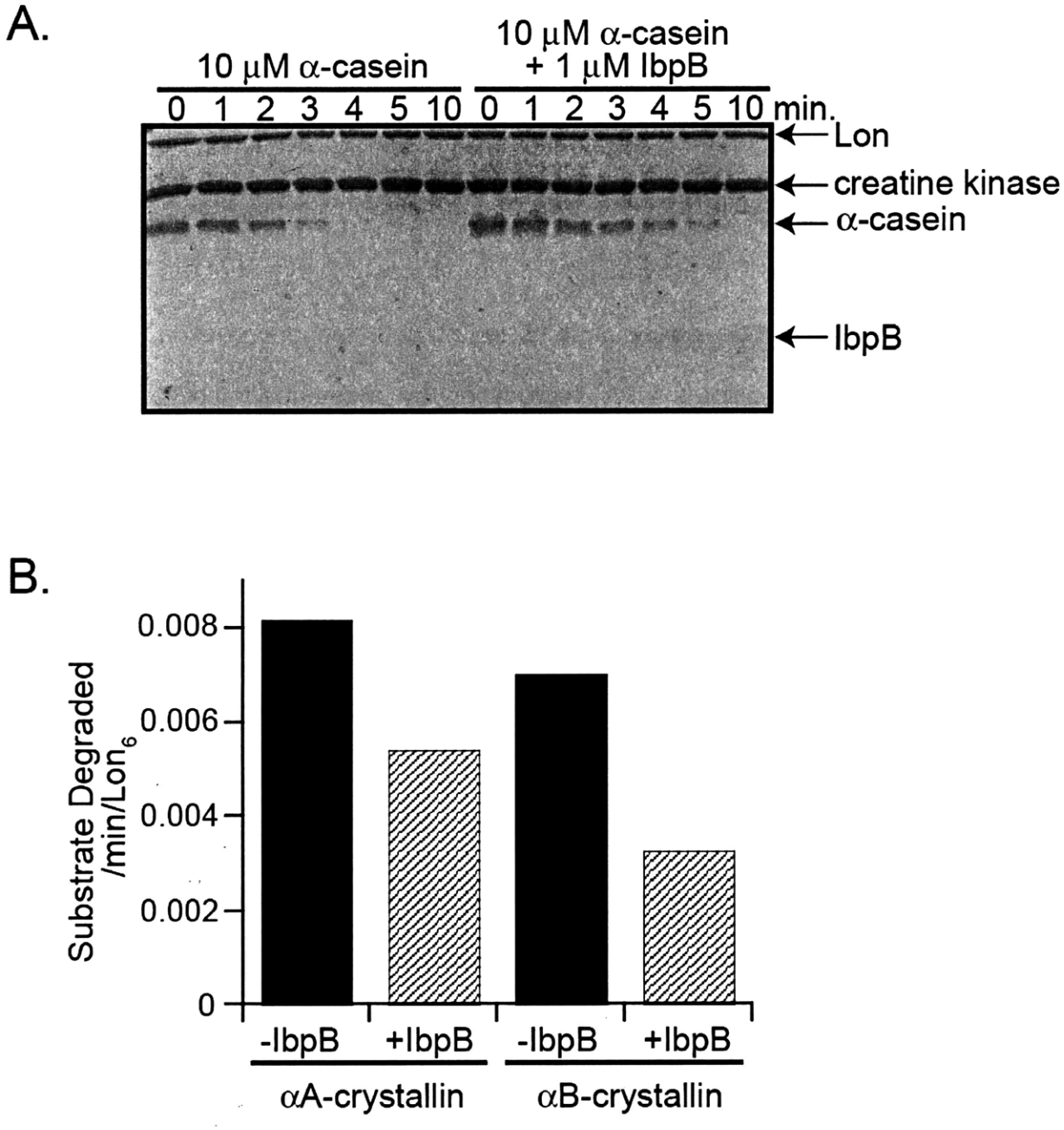

Figure 3.7: IbpB cannot facilitate the degradation of other Lon substrates.
(A) Coomassie stained gel showing the time course of degradation of 10 μM α-casein in the absence (left side) or presence (right side) of 1 μM IbpB by 300 nM Lon_6. (B) Degradation rate of 5 μM ^{35}S-labeled αA-crystallin (left side) or 5 μM ^{35}S-labeled αB-crystallin (right side) in the absence (solid bars) or presence (striped bars) of 5 μM IbpB by 600 nM Lon_6.

However, we saw no increase in the rate of degradation of these proteins in the presence of equimolar IbpB (Fig. 3.7B and data not shown). Together, these data strongly argue against the model that IbpB is an activator of Lon, at least not at the concentrations and under the experimental conditions used in these experiments. Furthermore, the observation that of the substrates we investigated, only IbpB activates IbpA degradation (Fig. 3.1) and that IbpB can only stimulate the degradation of IbpA (Chapter 2, Fig. 2.5 and Fig 3.7), strongly argue for the IbpA activation model. Thus, the mechanism of how IbpB facilitates IbpA degradation is most likely that IbpB is acting directly on IbpA to make IbpA a better substrate.

Discussion

In the present work we set out to investigate the mechanism by which IbpB facilitates IbpA degradation. We demonstrate that the N- and/or C-terminal tails of IbpB are necessary for IbpB-facilitated IbpA degradation, as the tail-less $IbpB^{\alpha}$ does not stimulate IbpA degradation (Fig. 3.1). The inability of $IbpB^{\alpha}$ to facilitate IbpA degradation was not solely due to the inability of $IbpB^{\alpha}$ to form higher-order oligomers, as chimeric $IbpB^{AT}$ retains the ability to form higher-order oligomers (Fig. 3.2B), but no longer facilitates IbpA degradation (Fig. 3.3B). We further show that IbpB does not stimulate the degradation of any other Lon substrate that we investigated (Fig. 3.7), suggesting that IbpB is making IbpA a better Lon substrate by directly and specifically acting on IbpA, not by acting as a general allosteric activator of Lon.

Dissecting the roles of the IbpB tails in the activation of IbpA degradation

Given our previous work demonstrating that the N- and C-terminal tails of the Ibps are important determinants of the rates of degradation of IbpA and IbpB (Chapter 2, Fig. 2.2), we were interested in whether the tails of IbpB were necessary for IbpB's ability to facilitate IbpA degradation. However, our result that $IbpB^{\alpha}$ could not facilitate IbpA degradation (Fig. 3.1) was inconclusive, since the tails of IbpB are known to affect the higher-order oligomerization of IbpB (Jiao *et al.*, 2005b). Therefore it was unclear if the inability of $IbpB^{\alpha}$ to facilitate IbpA degradation was due to the absence of its tails, or due to the inability of $IbpB^{\alpha}$ to form higher-order oligomers.

To differentiate between these two possibilities we constructed a chimeric version of IbpB containing the α-crystallin domain of IbpB, but the N- and C-terminal tails of IbpA ($IbpB^{AT}$). We found that this protein, while still able to form higher-order oligomers (Fig. 3.2B), and still a Lon substrate (Fig. 3.2C), was unable to facilitate IbpA degradation to any appreciable extent (Fig. 3.3B). Therefore higher-order oligomerization of a Lon substrate is not sufficient to facilitate IbpA degradation, and the tails of IbpB are critical determinants that enable IbpB-facilitated IbpA degradation.

Differentiating between models for how IbpB facilitates IbpA degradation

We previously demonstrated that IbpB facilitates IbpA degradation both *in vivo* and *in vitro* (Chapter 2, Fig. 2.5). Here we proposed two possible explanations for this phenomenon. One possibility was that IbpB is acting as an allosteric activator of Lon, making Lon a better enzyme and thereby making Lon better able to degrade IbpA. We called this the Lon activation model. This was an attractive hypothesis, because other Lon substrates are known to allosterically activate Lon (Waxman and Goldberg, 1986). Furthermore, Gur and Sauer recently demonstrated that different degradation tags can "tune" the speed and efficiency with which Lon degrades its substrates (Gur and Sauer, 2009), again suggesting a model for how IbpB might accelerate IbpA degradation. However, several lines of evidence presented here argue against the Lon activation model.

First, given that the N- and C-terminal tails of IbpB were required for IbpB to facilitate IbpA degradation, if IbpB was acting as an allosteric activator of Lon, we might have

expected that the tails of IbpB alone would enhance the ATPase activity of Lon, or even that the tails alone might facilitate IbpA degradation. Although the N- and C-terminal tail peptides of IbpB alone enhanced the ATPase rate of Lon, we also observed that the IbpA tail peptides alone enhanced Lon's ATPase rate (Fig. 3.5), suggesting that this activation of Lon ATPase activity by the IbpB tail peptides cannot explain IbpB-stimulated IbpA degradation. Furthermore, we saw no evidence that the tail peptides of IbpB alone facilitate the degradation of IbpA (Fig. 3.6). However, the most convincing result that argues against the Lon activation model is that under our experimental conditions, IbpB does not enhance the degradation of any other Lon substrate we investigated, other than IbpA (Fig. 3.7). If IbpB was a general activator of Lon, IbpB should be able to facilitate the degradation of other Lon substrates.

Given these results we favor the model whereby IbpB facilitates IbpA degradation by acting directly on IbpA, thereby making IbpA a better substrate. The best evidence for this model so far is that the ability of IbpB to facilitate the degradation of IbpA is completely specific, both from the side of IbpB (the activator protein), and from the side of IbpA (the activated protein). That is, of the substrates we investigated, IbpB only activates IbpA degradation (Chapter 2, Fig. 2.5 and Fig. 3.7). In addition, of the substrates we investigated, IbpA degradation is only activated by IbpB (Fig. 3.1 and data not shown). Even $IbpB^{AT}$, which forms higher-order oligomers, is itself a Lon substrate, and is identical to IbpB except in its N- and C-terminal tail regions, is unable to activate IbpA degradation (Fig. 3.2B,C and 3.3B). This specificity argues for the idea that IbpB is acting directly through IbpA, and against the idea that IbpB is acting in a

more general way through Lon. Presumably IbpB exerts its effect on IbpA through eliciting a conformational change in IbpA, thereby making IbpA easier to degrade, rather than by making IbpA better-recognized by Lon. Although formally possible, it is unlikely that IbpB is helping IbpA to bind to Lon, given that Lon recognizes IbpA and IbpB equally well, based on the similarity of the K_M values for IbpA and IbpB degradation (Chapter 2, Fig. 2.1 and Table 2.1). Therefore, it is more likely that the presumed conformational change that IbpB elicits on IbpA makes IbpA easier to unfold or translocate by Lon. However, more work must be done to uncover the exact nature of this conformational change.

The significance of the activation of IbpA degradation as a mode of regulation

There are numerous examples in biology of differential degradation of protein complexes, as compared to the degradation of the individual components that make up these complexes. But in most cases, being in a complex inhibits the degradation of the component proteins, while the uncomplexed components are sensitive to degradation. For example, the *Saccharomyces cerevisiae* MAT transcription factors α2 and a1 are stable in their hetero-dimeric form, but are degraded rapidly in the absence of their partner protein (Johnson *et al.*, 1998). Similarly, the mitochondrial processing peptidase is made up of two subunits, each subunit alone is a substrate of the mitochondrial Lon protease, but when in its functional complex, the subunits are resistant to Lon degradation (Ondrovicová *et al.*, 2005). Finally, in human cells, the binding of Hdmx to p53 (a transcriptional repressor) prevents the ubiquitylation of p53, thereby stabilizing it

(Stad *et al.*, 2000). Again, in each of these cases, degradation of a proteolytic substrate is inhibited by being in a complex with another protein.

The case of IbpA and IbpB is unusual because it involves an interaction between two proteins that accelerates the degradation of one of the proteins. One similar example of this kind of regulation is that of UmuD in *E. coli.* Active UmuD is involved in the SOS response and is part of the DNA replication machinery involved in bypassing lesions that would normally halt DNA replication (Gonzalez and Woodgate, 2002). In its intact form, UmuD is inactive and an excellent Lon substrate (Gonzalez *et al.*, 1998). However, under stress conditions, UmuD is cleaved, resulting in UmuD' which is active and no longer a Lon substrate. (Frank *et al.*, 1996). Therefore, the fact that intact UmuD is degraded so much faster than cleaved UmuD' is a critical mode of UmuD regulation and has an important physiological consequence. Similarly, we propose that this regulation of IbpA degradation, whereby IbpA is degraded much faster both *in vivo* and *in vitro* in the presence of IbpB, happens because degradation of IbpA in the presence of IbpB has an important physiological role. The significance of the degradation of the Ibps for protein quality-control will be explored in Chapter 4.

Materials and Methods

Proteins:

Lon, Ibps, and α-crystallin proteins were purified using the same methods as in Chapter 2. The isolated α-crystallin domains of IbpA and IbpB consisted of residue numbers 40-123 and 36-121 respectively. $IbpB^{AT}$ consisted of residues 1-39 of IbpA, followed by residues 36-121 of IbpB, followed by residues124-137 of IbpA. A methionine was added to the N-terminus of all of these constructs to facilitate labeling with ^{35}S-methionine.

Degradation *in vitro*:

Degradation of ^{35}S-labeled substrates *in vitro* was performed using 600 nM Lon_6 in 50 mM Tris-HCl (pH 8.0), 15 mM $MgCl_2$, 5 mM KCl, 1 mM DTT, 2% DMSO, 25 mM HEPES-KOH (pH 8.0), 400 mM potassium glutamate, 10% sucrose, and 0.5 mM BME. Lon and substrate were incubated at 37 °C for 2 min before the addition of the ATP-regeneration mix (32 mM ATP, 400 mg/ml creatine kinase, 40 mM creatine phosphate). Time points were taken every 5 min for 30 min. Reactions were quenched in 10% trichloroacetic acid (TCA). The TCA-insoluble material was removed by centrifugation and ^{35}S radioactivity in the supernatant was measured in a Tri-Carb liquid scintillation counter (Perkin Elmer). The fraction of substrate that had been degraded at each time point was calculated by dividing the TCA-soluble counts by the total counts in an equal volume of each reaction.

α-casein degradation was performed in a similar way, except 10 μM unlabeled α-casein (Sigma) was incubated with 300 nM Lon_6 under the buffer conditions described above.

After addition of the ATP regeneration mix, 10 μL of the reaction mixture was taken at the indicated time points and immediately added to 5 μL 3x SDS sample buffer and boiled for 5 minutes. The reactions were run on a 12.5% SDS PAGE gel and stained with coomassie.

Determination of oligomeric-state by gel filtration chromatography:

50 μL of IbpB or $IbpB^{AT}$ (50 μM each) was loaded onto a GE Health Sciences Superdex 200 PC 3.2/30 FPLC column (pre-equilibrated with a buffer containing 50 mM HEPES-KOH [pH 8.0] and 300 mM potassium glutamate). Peaks were detected by measuring the absorbance at 280 nm. A mix of molecular weight standards (BioRad), which included thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa) and vitamin B_{12} (1.35 kDa), were run under the same conditions.

Peptides

IbpA and IbpB tail peptides were synthesized using standard solid phase peptide synthesis techniques with an Aaptec Apex 396 peptide synthesizer. The N- and C-terminal tail peptides of IbpA consisted of residues 1-39 and 124-137 respectively. The N- and C-terminal tail peptides of IbpB consisted of residues 1-35 and 122-142 respectively.

Lon ATPase rates

The ATPase rate of Lon was measured using a coupled ATPase assay as described (Nørby, 1988). Briefly, 150 nM Lon_6 was incubated alone or with the indicated

peptide/protein at 37°C for 2 minutes in 50 mM Tris-HCl (pH 8.0), 15 mM $MgCl_2$, 5mM KCl, 1 mM DTT in a total reaction volume of 95 μL. 5 μL of the ATPase mix (20 mM NADH, 150 mM phosphoenol pyruvate, 0.5 mg/mL lactate dehydrogenase, 50 mM ATP, 1 mg/mL pyruvate kinase) was added and mixed well. Absorbance was monitored at 340 nm for 5 minutes at 37°C in a Molecular Devices Spectramax 384plus spectrophotometer.

Chapter Four

Degradation of IbpA and IbpB by Lon facilitates the refolding of Ibp-bound aggregated proteins

Abstract

Small heat-shock proteins (sHSPs) are α-crystallin domain-containing molecular chaperones that bind to damaged proteins and keep them in a refolding-competent state. The *E. coli* genome encodes two sHSPs, IbpA and IbpB, which are substrates of the AAA+ protease Lon. Recent data suggest that Lon can degrade client-bound Ibps *in vivo*. Here we investigate the significance of this observation in protein quality-control processes. We test two hypotheses: one hypothesis is that the Ibps deliver client proteins to Lon and are degraded in the process. The second hypothesis is that degradation of client-bound Ibps disengages the Ibps from their client proteins, allowing refolding of these clients. Our results argue against the model that the Ibps deliver client proteins to Lon for degradation, as we were unable to find any evidence for client protein degradation. However, we find that the presence of Lon in an Ibp-bound luciferase refolding assay leads to a two-fold increase in luciferase activity over the presence of the disaggregase ClpB. Our data therefore suggest that the role of Lon-dependent degradation of client-bound Ibps is to facilitate the refolding of clients, thereby reinforcing the importance of Lon-dependent Ibp degradation in the protein quality-control process.

Introduction

Small heat-shock proteins (sHSPs) are a family of molecular chaperones that bind damaged and unfolded client proteins to prevent their irreversible aggregation, and to keep them in a refolding-competent state (Narberhaus, 2002). The *E. coli* genome encodes two sHSPs, *ibpA* and *ibpB*, which are transcribed from the same operon (Allen *et al.*, 1992). IbpA and IbpB have been implicated in resistance to heat-shock, oxidative stress, and copper-induced stress (Kitagawa *et al.*, 2002; Matuszewska *et al.*, 2008). However, IbpA and IbpB do not work alone. Refolding of the Ibp client proteins requires the concerted effort of proteins from other branches of the quality-control network, including the ClpB disaggregase and the DnaK/DnaJ/GrpE (DnaKJE) and GroEL/ES refolding chaperones (Ratajczak *et al.*, 2009; Veinger *et al.*, 1998).

We recently demonstrated that IbpA and IbpB are substrates of the AAA+ (ATPases associated with various cellular activities) protease Lon (Chapter 2), a protease known to be important for the degradation of unfolded and damaged proteins *in vivo* (Kowit and Goldberg, 1977; Maurizi *et al.*, 1985). Our data further suggested that Lon degrades client-bound Ibps *in vivo* (Chapter 2, Fig. 2.5B). This finding implies that collaboration between Lon and the Ibps might be important for optimal protein quality-control. However, the means by which the degradation of the Ibps by Lon participates in proper protein quality-control is unclear. There are at least two possibilities for how degradation of client-bound Ibps by Lon might enhance protein quality-control (Fig. 4.1). One possibility is that the Ibps deliver their client proteins to Lon and that in the process

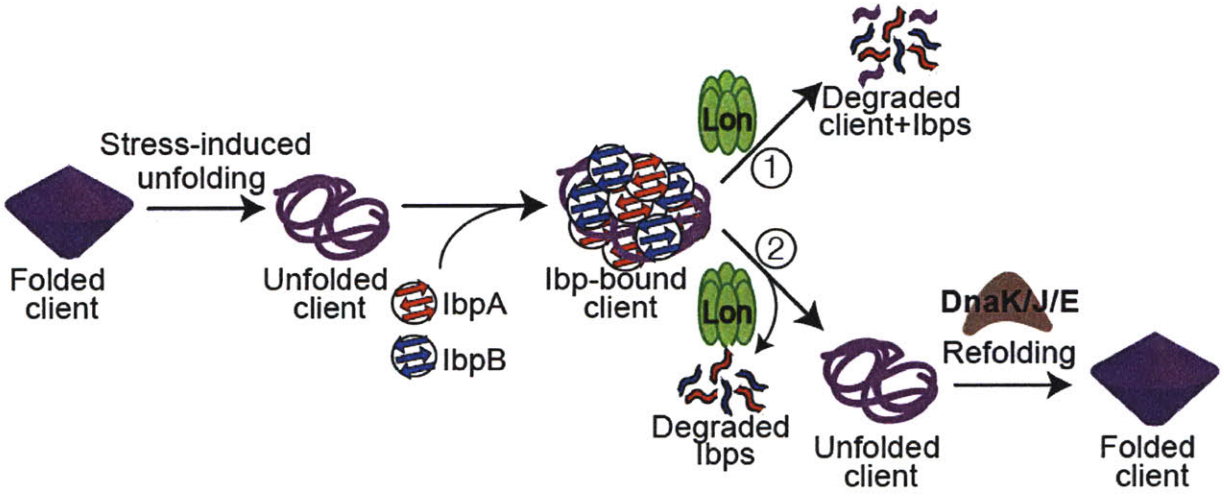

Figure 4.1: Two proposed roles for the degradation of client-bound Ibps.
Model of the two proposed roles for the degradation of client-bound Ibps: (1) Ibps deliver client proteins to Lon, leading to the degradation of both the client protein and the Ibps. (2) Degradation of client-bound Ibps removes the Ibps from their client protein, facilitating the refolding of the client protein by refolding chaperones such as DnaKJE.

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of degrading these client proteins, Lon degrades the Ibps (Fig. 4.1(1)). In this scenario, Ibps facilitate protein quality-control by binding to damaged proteins and delivering them to Lon, but are degraded in the process. A second possibility is that the degradation of Ibps by Lon facilitates the refolding of client proteins by removing bound Ibps (Fig. 4.1(2)). In this scenario, Ibp degradation facilitates protein quality-control by helping the refolding chaperones access Ibp-bound client proteins. Previously, this function of removing Ibps from bound client proteins was thought to be accomplished by the ClpB disaggregase, and indeed, ClpB facilitates the DnaKJE-mediated refolding of Ibp-bound aggregated luciferase, a model client protein (Ratajczak *et al.*, 2009). However, given our observation that Lon degrades client-bound Ibps (Chapter 2, Fig. 2.5), it seemed

likely that Lon might also play a role in this process of removing the Ibps from their clients.

In this work we use a variety of *in vitro* assays to investigate the functional significance of the degradation of client-bound Ibps to protein quality-control. Furthermore, we find that the most plausible explanation for the significance of the degradation of client-bound Ibps is that this degradation facilitates client refolding by removing bound Ibps.

Results

Assay for the client-bound state of the Ibps

We previously demonstrated that Lon degrades client-bound Ibps *in vivo* (Chapter 2, Fig. 2.5). We hypothesized that this degradation might have one of two biological functions, as detailed in the introduction (Fig. 4.1). The first model is that Ibps deliver their bound client proteins to Lon for degradation, and are themselves degraded in the process (Fig. 4.1(1)). The second model is that degradation of the Ibps releases client proteins so that they can be refolded by the DnaKJE or GroEL/ES refolding chaperones (Fig. 4.1(2)). To investigate the importance of the degradation of client-bound Ibps *in vitro*, we needed an assay that would allow us to determine the client-bound state of the Ibps. For this we used the ability of the Ibps to suppress client protein aggregation as an *in vitro* assay for the client-bound state of the Ibps.

Like all functional sHSPs, the Ibps suppress the aggregation of a variety of proteins (Matuszewska *et al.*, 2005; Ratajczak *et al.*, 2009; Shearstone and Baneyx, 1999). Interestingly, whether both IbpA and IbpB are required for this suppression seems to depend on a variety of factors, including the conditions under which the aggregation is performed, as well as on the client protein itself. Similar to what has been shown previously, we observed that IbpB alone only provided a modest suppression of the aggregation of malate dehydrogenase (MDH), a model client protein, whereas IbpA alone suppressed MDH aggregation to a much greater extent (Fig. 4.2). There was no additive effect in the suppression of MDH aggregation in the presence of both IbpA and

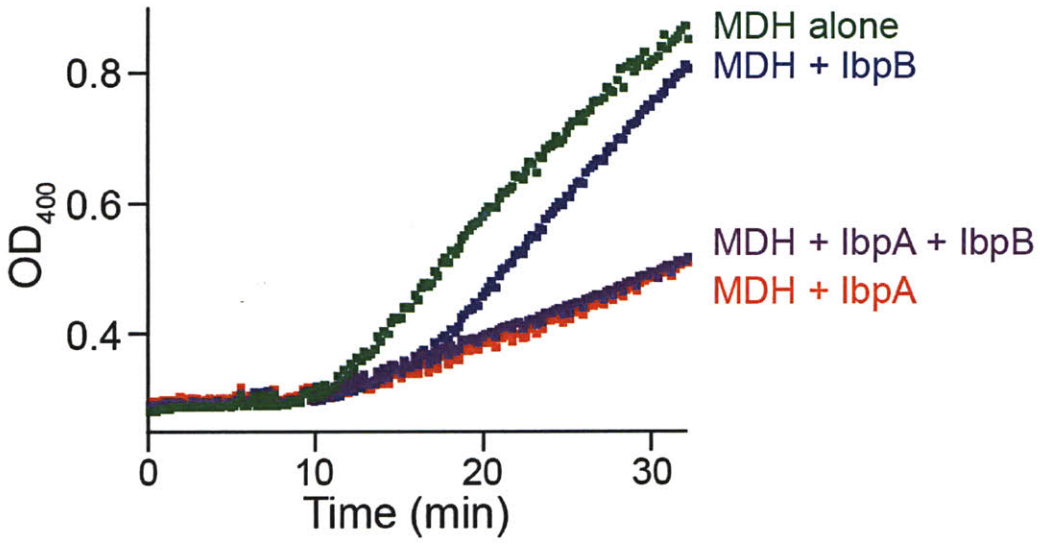

Figure 4.2: IbpA suppresses MDH aggregation to a much greater extent than does IbpB.
20 μM MDH was incubated alone (green) or with 10 μM IbpA (red), 10 μM IbpB (blue) or 10 μM each of IbpA and IbpB (purple) in a UV-Vis plate reader at 48°C for 30 minutes. Aggregation was measured by light scatter at OD_{400}.

IbpB (Fig. 4.2). The most likely explanation for these results is that IbpA is in much closer functional contact with MDH than is IbpB.

Client proteins affect IbpA degradation, but not IbpB degradation

Using this ability of the Ibps to suppress MDH aggregation as a read-out for being client-bound, we were able to indirectly test whether IbpA and/or IbpB deliver bound MDH to Lon for degradation. Specifically, we measured the rate of Lon-dependent degradation of ^{35}S-labeled IbpA in the presence of equimolar unlabeled IbpB, or ^{35}S-labeled IbpB in the presence of equimolar unlabeled IbpA, after aggregation with increasing amounts of MDH. Ibp degradation rates were determined by measuring the appearance of TCA-soluble ^{35}S-labeled peptides over time. We added the unlabeled partner Ibp to each reaction because we previously demonstrated that IbpA and IbpB

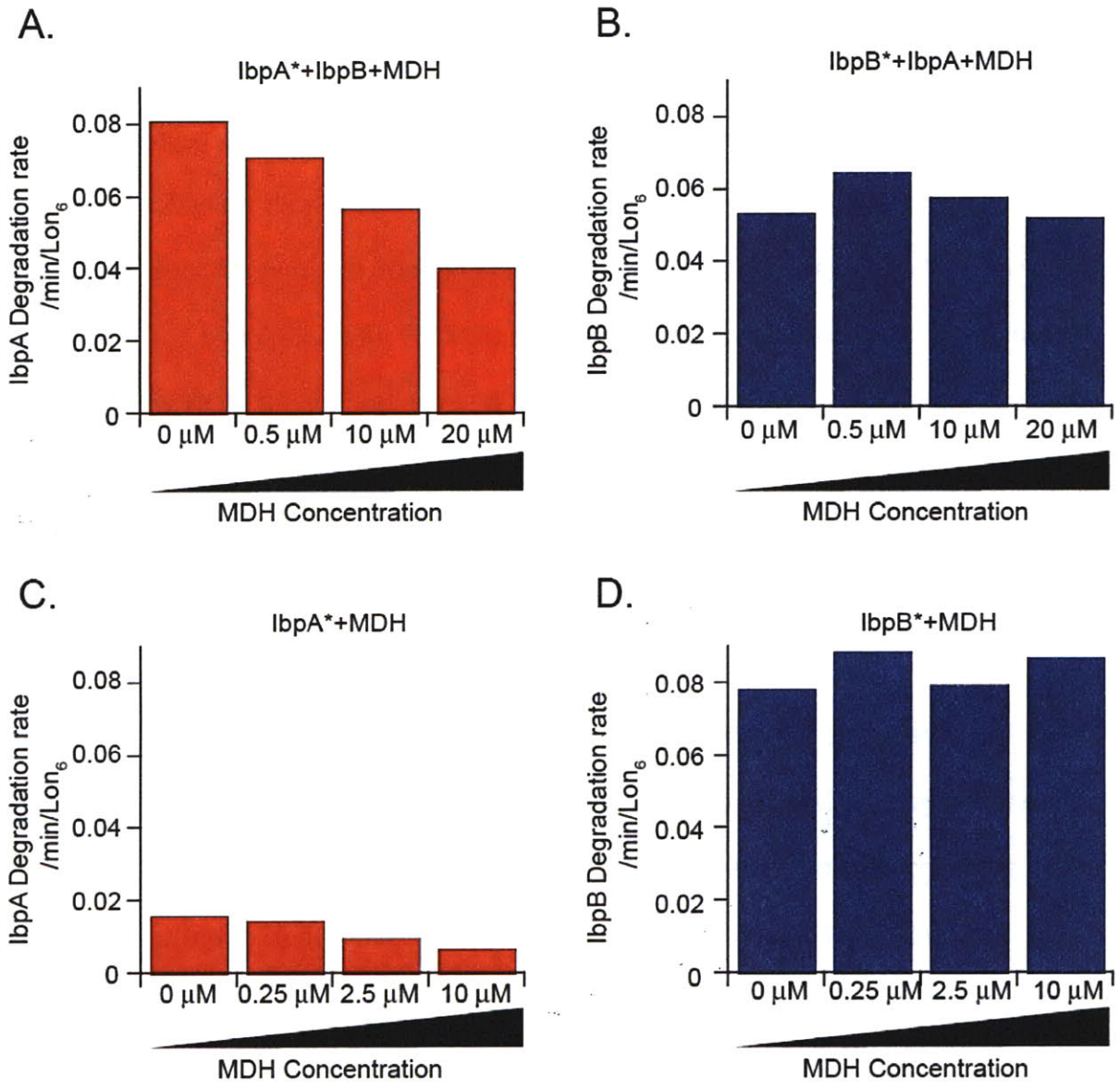

Figure 4.3: The presence of the MDH client protein affects IbpA degradation but not IbpB degradation.
(A) 10 μM ^{35}S-labeled IbpA and 10 μM unlabeled IbpB or (B) 10 μM ^{35}S-labeled IbpB and 10 μM unlabeled IbpA were incubated with 0, 1, 20, or 40 μM MDH at 48°C for 30 minutes. The reactions were then diluted by half and 600 nM Lon_6 was added for the degradation reaction, which was performed at 37°C. The rate of degradation was determined by measuring the appearance of TCA-soluble ^{35}S-labeled peptides over time in a scintillation counter. (C) and (D) Experiments were performed as in (A) and (B), but without adding the unlabeled IbpA/IbpB. Also, 0, 0.5, 5, or 20 μM MDH was used in the aggregation reaction.

cooperate in their degradation by Lon (Chapter 2, Fig. 2.5), and we therefore reasoned that IbpA and IbpB may also cooperate to deliver client proteins to Lon. If the Ibps deliver MDH to Lon for degradation, it is likely that the Ibps would be degraded slower in the presence of MDH, as the Ibps would have to compete with MDH for degradation. However, only the rate of IbpA degradation, and not the rate of IbpB degradation, decreased as we increased the concentration of MDH (Fig. 4.3A and B). These results indicate that Ibp-bound MDH is not degraded by Lon, because if MDH were degraded, we would expect degradation of both IbpA and IbpB to be inhibited, as both would have to compete with MDH for degradation by Lon. Thus these data are inconsistent with the hypothesis that the Ibps deliver the model MDH client protein to Lon for degradation.

To further investigate why IbpA degradation was slowed in the presence of increasing concentrations of MDH, we measured the rate of degradation of IbpA alone or IbpB alone after aggregating IbpA or IbpB with increasing concentrations of MDH. Again, we found that MDH affected the rate of IbpA degradation, but not the rate of IbpB degradation (Fig. 4.3C and D). Although as expected from our previous work (Chapter 2, Fig. 2.5), at all concentrations of MDH, the rate of degradation of IbpA was significantly slower in the absence of IbpB than in the presence of IbpB (Fig. 4.3C compared to Fig. 4.3A). Together, these results suggest that IbpA, but not IbpB, is in direct contact with the MDH client protein. Presumably, this interaction between IbpA and MDH leads to the slower degradation of IbpA. These results are also consistent with the result that IbpA suppresses MDH aggregation to a much greater extent than does IbpB (Fig. 4.2), which again suggests that only IbpA is in direct contact with MDH.

Interestingly, this architecture does not seem to be altered even when both IbpA and IbpB are present. That is, even in the presence of IbpA, IbpB still does not interact with MDH as determined by the observation that even in the presence of IbpA, the addition of increasing concentrations of MDH does not affect IbpB degradation (Fig. 4.3B). Therefore, IbpA does not seem to change the way IbpB interacts with client proteins. These data further support the idea that IbpA and IbpB have different activities in the

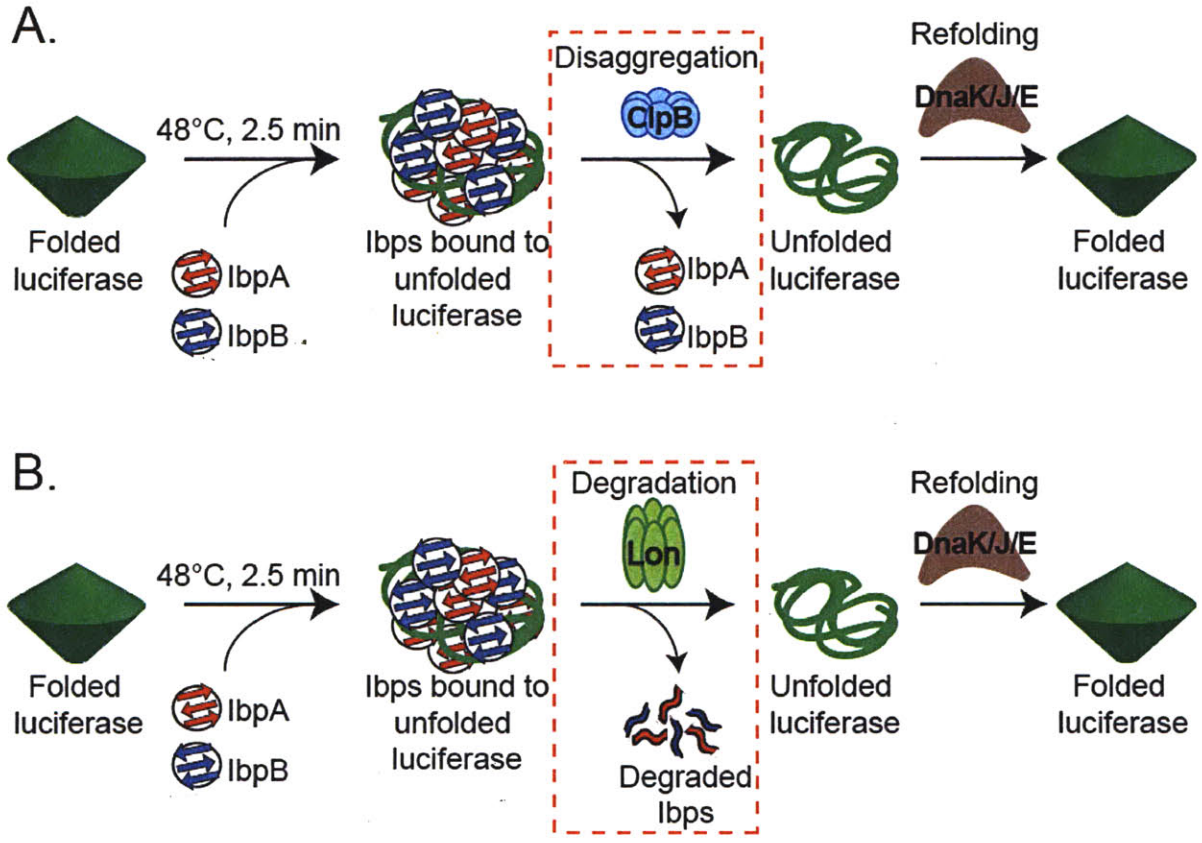

Figure 4.4: Schematic of the luciferase refolding assay
Schematic of the luciferase refolding assay and model for the involvement of ClpB (A) and Lon (B) in this process.

chaperoning of client proteins, and expand on this idea by suggesting that IbpA is unable to promote interaction between IbpB and client proteins.

Degradation of Ibps by Lon facilitates refolding of Ibp-bound client proteins.

Next we decided to directly test the hypothesis that degradation of client-bound Ibps by Lon facilitates the refolding of client proteins by the refolding chaperones (Fig. 4.1(2)). To do this we used a previously-published luciferase aggregation and refolding assay. This assay involves aggregating the model client protein luciferase in the presence of Ibps, and then reactivating the aggregated luciferase by adding ClpB, DnaK, DnaJ and GrpE, and monitoring reactivation by measuring luciferase activity over time (Fig. 4.4A) (Ratajczak *et al.*, 2009). ClpB is a disaggregase and is therefore thought to remove the Ibps from the aggregated luciferase, while DnaK is a refolding chaperone that works in conjunction with its co-chaperones DnaJ and GrpE to refold the luciferase (Genevaux *et al.*, 2007; Mogk *et al.*, 2003b). In particular, we were interested in whether Lon could substitute for ClpB (Fig. 4.4B), which presumably acts to disengage the Ibps from their clients (Mogk *et al.*, 2003b).

Surprisingly, we found that Lon reactivated luciferase to an even greater extent than ClpB. The activity of aggregated luciferase after incubation with Lon and DnaKJE for one hour was two-fold greater than the luciferase activity after incubation with ClpB and DnaKJE (Fig. 4.5A). Because Lon has been observed to have chaperone activity independent from its proteolytic activity (Suzuki *et al.*, 1997), we asked whether this enhancement of luciferase refolding was dependent on the proteolytic activity of Lon.

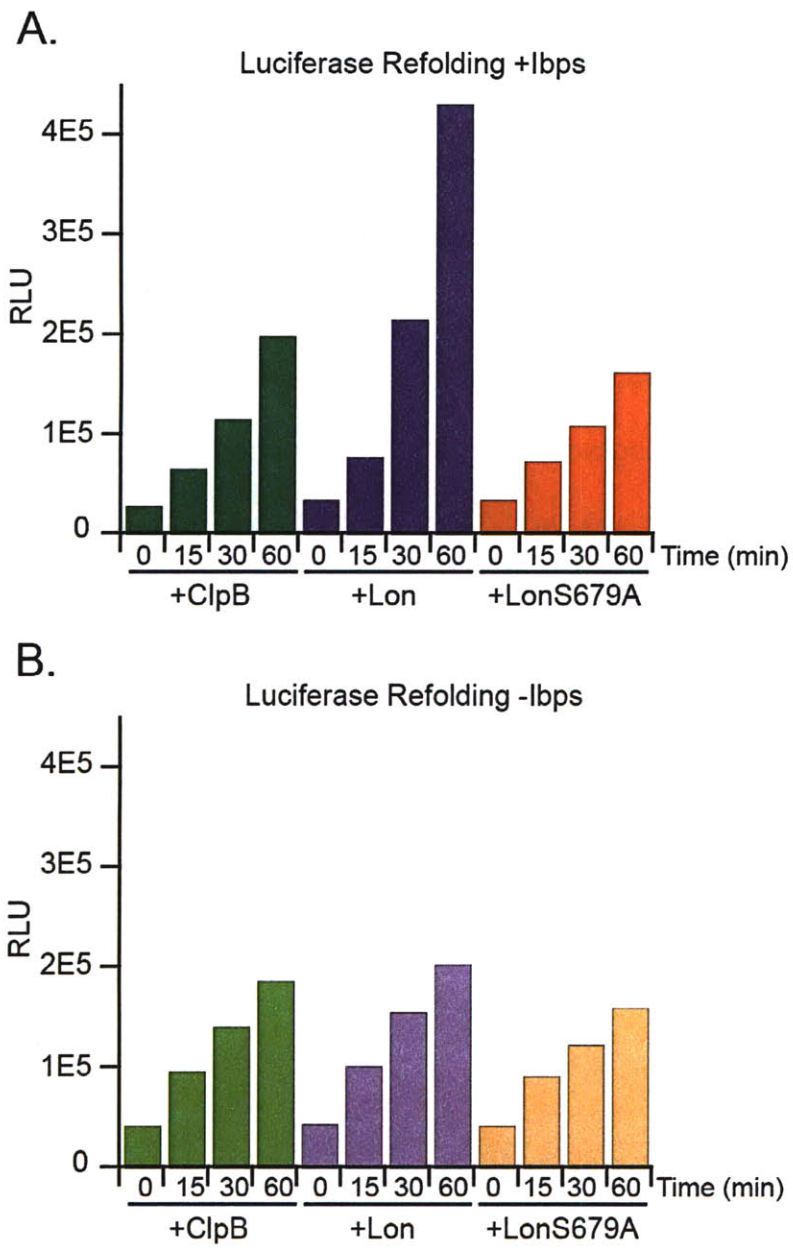

Figure 4.5: Lon facilitates the refolding of Ibp-bound luciferase
(A) Luciferase was inactivated by incubation at 48°C for 2.5 minutes in the presence of IbpA and IbpB, and then diluted into a refolding reaction containing ClpB, Lon, or LonS679A, as well as DnaK, DnaJ, and GrpE. Refolding was monitored by measuring luciferase activity over time (see materials and methods for details). (B) Experiments were performed as in (A) except that the Ibps were omitted from the initial luciferase aggregation step.

We found that the proteolytically inactive mutant, LonS679A, was unable to enhance luciferase refolding over that observed with ClpB (Fig. 4.5A). These data indicate that the ability of Lon to enhance luciferase refolding is dependent on its proteolytic activity, and suggest that degradation of the Ibps by Lon facilitates client protein refolding.

To further test this model, we measured the reactivation of luciferase that had been aggregated in the absence of Ibps. If degradation of the Ibps by Lon is the mechanism of Lon-enhanced luciferase refolding, we would expect that this enhancement in refolding would depend on the presence of Ibps in the initial aggregation reaction. Indeed, when Ibps were absent from the initial luciferase aggregation reaction, Lon was unable to enhance luciferase reactivation over ClpB-mediated reactivation (Fig. 4.5B). Together, these data suggest that Lon facilitates the refolding of Ibp-bound luciferase by degrading the bound Ibps. Furthermore, these data suggest that the significance of the degradation of the client-bound Ibps by Lon *in vivo* is that this degradation facilitates client-protein refolding.

Discussion

The question of how sHSPs are removed from their client proteins such that these clients can be refolded was one that persisted for many years, until the discovery that ClpB greatly facilitates the refolding of sHSP-bound client proteins (Mogk *et al.*, 2003b). Since this discovery, the assumption has been that in bacteria, ClpB mediates the removal of Ibps from client proteins, and that once the clients have been disengaged they are refolded, for example, by the DnaKJE machinery (Ratajczak *et al.*, 2009). Here we demonstrate that Lon can take the place of ClpB in this disengagement step, and thereby provide a biological rationale for the degradation of the Ibps by Lon.

The Ibps do not deliver aggregated MDH to Lon

Our previous work suggested that Lon degrades client-bound Ibps *in vivo* (Chapter 2 Fig. 2.5). Therefore the main goal of this work was to gain insight into the mechanistic significance of client-bound Ibp degradation to protein quality-control. We hypothesized that either the Ibps were delivering client proteins to Lon and being degraded in the process, or that degradation of client-bound Ibps was freeing the Ibp-bound clients, enabling the clients to be refolded by the refolding chaperones (Fig. 4.1). To begin to investigate these questions, we sought to determine how being bound to client-proteins affected the degradation of the Ibps, using the ability of the Ibps to suppress MDH aggregation as a read-out for the client-bound state of the Ibps.

We reasoned that if the Ibps deliver clients (MDH) for degradation, then in the presence of MDH, Ibp degradation should be slower, as the Ibps would have to compete for

degradation with MDH. However, when we measured the rate of degradation of ^{35}S-labeled IbpA in the presence of unlabeled IbpB and vice versa after aggregating the Ibps with MDH, we found that MDH only slowed the degradation of IbpA, not the degradation of IbpB (Fig. 4.3A and B). These results were inconsistent with the hypothesis that the Ibps deliver MDH for degradation, because if either of the Ibps delivered MDH to Lon, we would expect the degradation of both Ibps to be slowed, since both Ibps should have to compete with MDH for degradation by Lon. Therefore, these results suggest that Lon does not degrade MDH, and these data are inconsistent with the first model, that the Ibps deliver bound client proteins to Lon for degradation (Fig. 4.1(1)). Of course, a reduction in the degradation rate of MDH-bound Ibps is only an indirect assay for MDH degradation, and the ability of MDH to compete for degradation with the Ibps in a detectable manner would depend on the kinetic parameters of MDH degradation. Therefore, it would be more informative to directly measure the degradation of Ibp-bound clients. Theoretically this could be accomplished by labeling MDH with tritium, in which case the degradation of ^{35}S-labeled IbpA or IbpB could be measured simultaneously with the degradation of ^{3}H-labeled MDH. These experiments are currently ongoing.

On the other hand, the data that MDH affects IbpA degradation but not IbpB degradation are consistent with our observation that IbpA is much better than IbpB at suppressing MDH aggregation (Fig. 4.2), which suggests that IbpA binds MDH much better than does IbpB. Presumably, the slower degradation of IbpA in the presence of increasing concentrations of MDH (Fig. 4.3A and C), is due to this ability of IbpA to

interact with MDH. Because IbpB does not functionally interact with MDH (Fig. 4.2), IbpB degradation is not affected by the presence MDH (Fig. 4.3B and D). Furthermore, this result that IbpB degradation is unaffected by the addition of increasing concentrations of MDH, both in the presence and absence of IbpA (Fig. 4.3B and D) is particularly interesting in the context of the architecture of the IbpA•IbpB•client complex, and suggests that even in the presence of IbpA, IbpB does not contact the client in a manner that slows its degradation. Therefore these data argue against any large-scale rearrangement of the IbpA-client complex in the presence of IbpB, and further support the idea that at least for MDH, IbpA is primarily responsible for suppressing the aggregation of this client, and IbpB most likely has a different role.

Lon facilitates the refolding of aggregated luciferase

Our second model for the significance of the degradation of client-bound Ibps was that this degradation is a way to remove Ibps from the client protein, thereby facilitating the refolding of client proteins by cellular refolding chaperones. It had been thought that ClpB fulfilled this function of disengagement of the Ibps from clients (Mogk *et al.*, 2003b). But given our observation that Lon degrades client-bound Ibps (Chapter 2, Fig. 2.5), it seemed reasonable that Lon might also have a role in this process. To test this model we used a luciferase refolding assay to determine if Lon could substitute for ClpB in its ability to facilitate the refolding of Ibp-bound aggregated luciferase (Fig. 4.4). In fact, the refolding of Ibp-bound luciferase in the presence of Lon was about twice as effective as in the presence of ClpB (Fig. 4.5A). Furthermore, this ability of Lon to accelerate luciferase refolding as compared to ClpB was dependent both on the

proteolytic activity of Lon and on the presence of Ibps in the initial luciferase aggregation reaction (Fig. 4.5A and B). The most likely explanation for these results is that the degradation of the luciferase-bound Ibps by Lon enhances luciferase refolding. This idea could be explicitly tested by measuring the degradation of luciferase-bound Ibps, and the refolding of Ibp-bound luciferase in parallel reactions. Nevertheless, our results provide a possible explanation for why client-bound Ibps are degraded *in vivo*, namely that this degradation facilitates the refolding of client proteins.

It is particularly intriguing that the refolding of aggregated luciferase was twice as effective in the presence of Lon as in the presence of ClpB (Fig. 4.5A). One explanation for this observation is that degradation of the Ibps prevents them from rebinding client proteins, which may inhibit the refolding process. In the context of the crowded cellular environment, it is perhaps more advantageous to degrade client-bound Ibps, rather than to simply disengage them, as disengagement could lead to the rapid rebinding of the Ibps to unfolded client proteins, inhibiting client protein refolding. However, more work will need to be done to understand the different contributions of ClpB and Lon to the refolding of Ibp-bound client proteins *in vivo*.

The results presented here suggest that the degradation of Ibps by Lon is indeed functional and serves to aid in the refolding of client proteins (Fig. 4.1(2)). We previously demonstrated that the degradation of IbpB alone is 15-fold faster than that of IbpA alone (Chapter 2, Fig. 2.1), and that the presence of equimolar IbpB enhances IbpA degradation 7-fold (Chapter 2, Fig. 2.5). However, the results reported here suggest that

for MDH, predominantly IbpA is in functional contact with this client. Therefore, an intriguing question that remains is whether the role of IbpB might be to enhance IbpA degradation, and whether this enhancement might be important for Lon's ability to facilitate the refolding of Ibp-bound substrates. Answering this question is critical for a thorough understanding of the importance of the degradation of client-bound Ibps in protein quality-control, and is currently under active investigation.

Materials and Methods

Proteins:

Lon, as well as unlabeled and ^{35}S-labeled IbpA and IbpB were purified using procedures detailed in Chapters 2 and 3. LonS679A was purified in the same way as wild-type Lon. ClpB was purified using a method modified from Malakhov *et al.* A His-SUMO fusion of ClpB was cloned downstream of the T7 promoter and expressed in *E. coli* BL21 cells. Two 1 L cultures were grown in LB broth at 37 °C, induced at OD_{600} 0.8 with 0.5 mM IPTG, grown for an additional three hours at 30 °C, harvested, and stored at -80 °C until purification. Cells were thawed, resuspended in 30 mL of lysis buffer (50 mM HEPES-KOH [pH 8.0], 1 mM BME, 500 mM potassium glutamate) and 1 μL of the nuclease Benzonase (Novagen) was added. The cells were lysed in a cell disruptor (Constant Systems) at 25 MPa, and the lysate was cleared by centrifugation at 30000 x g for 20 minutes. The supernatant was incubated with 2 mL Ni-NTA beads (Qiagen) for 30 min at 4 °C. The beads were packed in a column, rinsed with 500 mL LG-5 buffer (50 mM HEPES-KOH [pH 8.0], 1 mM BME, 400 mM potassium glutamate, 5 mM imidazole) followed by 500 mL LG-20 buffer (identical to LG-5 buffer except with 20 mM imidazole). The protein was eluted with 12 mL elution buffer (50 mM HEPES-KOH [pH 8.0], 1 mM BME, 400 mM potassium glutamate, 500 mM imidazole, 10% glycerol). Peak fractions were pooled and dialyzed overnight against 2 L of dialysis buffer 1 (50 mM HEPES-KOH [pH 8.0], 10% glycerol, 200 mM potassium glutamate, 1 mM BME). The His-SUMO domain was cleaved off by incubating the dialyzed protein with 10 μL of ULP protease (purified as in Malakov *et al.*) for 2.5 hours at 37 °C. The protein was then flowed over 2 mL packed Ni-NTA beads, and the flow-through fraction was collected,

concentrated and applied to a Superose 6 column, pre-equilibrated with ClpB buffer (20 mM Tris-HCl [pH 8.0], 5 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT, 0.5 mM EDTA, 20% glycerol). Peak fractions were pooled and concentrated.

DnaK, DnaJ, and GrpE were purchased from Assay Designs. MDH was purchased from Roche. Luciferase was purchased from Promega.

MDH aggregation suppression assay:

MDH was dialyzed against 1X PBS overnight before use. 20 μM MDH was incubated either alone or with the indicated concentrations of IbpA and/or IbpB. The final buffer contained 0.3X PBS, 6 mM HEPES-KOH (pH 8.0), 2.4% sucrose, and 72 mM potassium glutamate. 20 μL of each reaction was put in a well of a 384 well plate, which was placed in a Molecular Devices SpectraMax5 plate reader, pre-warmed to 48°C. Aggregation was followed by monitoring light scatter at 400 nm for 30 minutes.

Degradation reactions:

To measure the degradation of MDH-bound Ibps, MDH was first pre-aggregated with the Ibps as follows. 10 μM ^{35}S-labeled IbpA or IbpB was incubated with 0, 0.5, 5, or 20 μM MDH, which had been dialyzed overnight against 1X PBS. When both IbpA and IbpB were present, they were incubated with 0, 1, 20 or 40 μM MDH. The aggregation reaction was carried out in 0.3X PBS, 5 mM DTT, at 48°C for 30 minutes in a plate reader so that aggregation could be monitored as detailed above. For the degradation reaction, the aggregation reactions were diluted two-fold and supplemented with 50 mM

Tris-HCl (pH 8.0), 15 mM $MgCl_2$, 5 mM KCl, 600 nM Lon_6, 2% DMSO and an ATP regeneration mix (32 mM ATP, 400 mg/mL creatine kinase, 40 mM creatine phosphate). Time points were taken every 5 min for 30 min. Reactions were quenched in 10% trichloroacetic acid (TCA). The TCA-insoluble material was removed by centrifugation and ^{35}S radioactivity in the supernatant was measured in a Tri-Carb liquid scintillation counter (Perkin Elmer). The fraction of substrate that had been degraded at each time point was calculated by dividing the TCA-soluble counts by the total counts in an equal volume of each reaction, and plotted. The rate of degradation was calculated by taking the slope of this line.

Luciferase refolding assay:

Luciferase aggregation was performed as follows: 1.5 μM luciferase was incubated with 2 μM IbpA and 30 μM IbpB in aggregation buffer (50 mM Tris-HCl [pH 7.5], 150 mM KCl, 20 mM magnesium acetate, and 5 mM DTT) at 48°C for 2.5 minutes, and then immediately placed on ice. For refolding, the aggregated luciferase was then diluted to 0.04 μM and incubated with 1.5 μM DnaK, 0.2 μM DnaJ, 0.3 μM GrpE and 1.5 μM $ClpB_6$, Lon_6, or Lon_6S679A in refolding buffer (50 mM Tris-HCl [pH 8.0], 15 mM $MgCl_2$, 5 mM KCl) and supplemented with an ATP regeneration mix (32 mM ATP, 400 mg/ml creatine kinase, 40 mM creatine phosphate). 5 μL of each reaction was aliquoted into 4 wells of a white-bottom 96-well plate, and incubated in a luminescence plate reader set to 30°C. At each time point, 50 μL of the luciferase assay reagent (Promega) was added and luminescence was detected.

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Chapter Five

Perspectives on the future of small heat-shock protein proteolysis

In this thesis I demonstrate that the *E. coli* sHSPs IbpA and IbpB are substrates of the AAA+ Lon protease. Unexpectedly, in exploring the degradation determinants of IbpA and IbpB, I found that Lon seems to recognize a structural feature of the folded α-crystallin domains of the Ibps, rather than a particular peptide motif. I also demonstrate that IbpB facilitates IbpA degradation by directly activating IbpA, rather than by acting as a general allosteric activator of Lon. Finally, I show that degradation of IbpA and IbpB by Lon facilitates the refolding of an aggregated Ibp-bound client protein. The results presented in this work therefore provide compelling evidence for a novel connection between the proteolytic branch and the sHSP branch of the protein quality-control network, which leads to more efficient refolding of Ibp-bound aggregated proteins. However, this work also suggests many important questions, and these unanswered questions will be the focus of my final chapter.

Toward a greater understanding of the relationship between IbpA and IbpB

One of our most intriguing findings was the observation that IbpB facilitates IbpA degradation. This was surprising because binding to partner or accessory proteins usually leads to the stabilization of protease substrates (see Chapter 3 Discussion). One way we can try to understand the biological relationship between IbpA and IbpB is as a variation on an established biological theme. And although interactions between protease substrates and other proteins usually lead to the protection of these substrates from degradation, one major exception is that of adaptor proteins. For example, the adaptor proteins SspB and ClpS facilitate the degradation of ssrA-tagged proteins and N-end rule proteins by the ClpXP and ClpAP proteases respectively (Erbse *et al.*, 2006;

Levchenko *et al.*, 2000). One could think of IbpB as a specialized adaptor protein for IbpA, perhaps in the vein of RssB, an adaptor which so far seems to only facilitate the degradation of the σ^S transcription factor (Zhou *et al.*, 2001). An even more similar situation is that of UmuD. As mentioned previously, the UmuD pro-protein is a Lon substrate, but the cleaved, active UmuD' is not (Gonzalez *et al.*, 1998). However, UmuD acts as an adaptor for UmuD', leading to its degradation by ClpXP (Neher *et al.*, 2003).

This hypothesis that IbpB is an adaptor for IbpA makes the most sense if IbpB does not have another function (for example, chaperone activity) on its own. As mentioned previously, the extent to which IbpB alone is able to act as a chaperone is highly dependent on the particular client protein and on experimental conditions. For example, IbpB alone seems to suppress the aggregation of the model client protein citrate synthase, but does not suppress the aggregation of luciferase (Ratajczak *et al.*, 2009; Shearstone and Baneyx, 1999). Therefore, more systematic *in vitro* experiments are needed to determine whether IbpB is truly functional as a chaperone *in vitro*, or whether its predominant function is to facilitate IbpA degradation. Alternatively, if a *ΔibpB* strain has a detectable mutant phenotype, another way to test this hypothesis would be to investigate whether ectopic degradation of IbpA is sufficient to suppress the *ΔibpB* phenotype. If this is the case, it would support the idea that the predominant function of IbpB is to facilitate IbpA degradation.

Perhaps even more compelling is to think about the degradation of IbpA and IbpB, not as a variation on an established biological theme, but as a paradigm for another way in

which degradation can be regulated. The idea that a gene duplication event might lead to two genes, the protein product of one which evolves to regulate the degradation of the other, is an intriguing one, and we may very well find more examples of this type of regulation in the future.

To further explore these hypotheses, it would be particularly interesting to know whether this ability of one sHSP to facilitate the degradation of another sHSP is conserved in other organisms. Specifically, does IbpB facilitate IbpA degradation in organisms that encode the intact *ibpAB* operon, such as species in the *Shigella* and *Salmonella* genera? What about in organisms that encode multiple sHSPs in different parts of the genome, such as *Bacillus subtilis* and *Bradyrhizobium japonicum*? In the context of sHSP biology, *B. japonicum* is a particularly fascinating species as these bacteria encode seven sHSPs in their genomes (Münchbach *et al.*, 1999), while other prokaryotes typically encode one or two sHSPs (Kappé *et al.*, 2002). The question of why *B. japonicum* encodes so many sHSPs, and how the different sHSPs are regulated post-translationally has not been explored. Our data that the Ibps are Lon substrates, and that IbpB facilitates IbpA degradation, could give insights into the regulation of other sHSPs in other organisms, especially those encoding multiple sHSPs.

Of course, given that so many prokaryotes and archaea encode only one sHSP in their genomes, control of sHSP function by cooperative degradation with a partner sHSP cannot be a universally conserved mechanism. Therefore it would also be interesting to investigate the mechanisms that control sHSP levels and function in organisms with

only one sHSP. Perhaps in the absence of a partner sHSP, degradation does not play as big of a role in the regulation of these sHSPs. Alternatively, perhaps there are other non-sHSP regulatory proteins that affect the degradation of these sHSPs.

What is the significance of sHSP degradation *in vivo*?

Many questions remain related to the *in vivo* significance of the degradation of Ibps by Lon. Given our *in vitro* results, which showed that IbpA and IbpB are degraded in the absence of client proteins (Chapter 2, Fig. 2.1 and 2.5), it is possible that one purpose of Ibp degradation is to maintain the appropriate levels of IbpA and IbpB in the cell. This might be particularly important during recovery from heat-shock, as during this response the levels of Ibp proteins increase 10-fold (Mogk *et al.*, 1999). However, we demonstrated that the Ibps are degraded even during prolonged exposure to heat-shock (Chapter 2, Fig. 2.5B), conditions under which the Ibps are bound to client proteins. These results, together with the result that IbpA degradation is controlled by the presence of IbpB (Chapter 2, Fig. 2.5A and B), strongly suggest that the degradation of the Ibps in general, and the degradation of client-bound Ibps in particular, play an important biological role, in addition to participating in Ibp homeostasis.

In Chapters 2 and 4 we suggested two possible functions for the degradation of client-bound Ibps in protein quality-control. One possibility was that Ibps deliver their client proteins to Lon and are themselves degraded in the process. The other possibility was that degradation of client-bound Ibps releases the clients to facilitate their refolding. This

role of disengaging Ibps from client proteins was previously thought to be accomplished by ClpB (Mogk *et al.*, 2003b). In Chapter 4 I demonstrated that degradation of client-bound Ibps facilitates refolding of the Ibp-bound client proteins. Our observation that aggregated luciferase is refolded twice as well in the presence of Lon as in the presence of ClpB strongly suggests that the role of Lon may be even more important than that of ClpB when it comes to the refolding of at least certain Ibp-bound clients. Furthermore, we did not observe any degradation of Ibp-bound MDH *in vitro*, and it has been demonstrated *in vivo* that over-expression of IbpA and/or IbpB protects recombinant proteins from degradation (Han *et al.*, 2004). These data argue against a role for Ibps in the delivery of bound client proteins for degradation.

Nonetheless, it is certainly possible that under different conditions, both Lon-mediated protein refolding and Ibp-assisted protein degradation occur *in vivo*. Since sHSPs bind and sequester unfolded and damaged proteins, they are in the perfect position to regulate whether client proteins should be refolded or degraded. Perhaps for some clients, the Ibps are degraded away by Lon, leaving the client protein intact for refolding by the refolding chaperones. This might be particularly important for large client proteins or clients that refold slowly, as degrading the bound-Ibps would prevent the Ibps from rebinding the partially-unfolded clients. But other clients may be delivered by the Ibps to Lon for degradation. This might be especially important under conditions of high environmental stress, when the refolding chaperones may be overwhelmed. Under these conditions it may be more advantageous for cells to target some unfolded proteins for degradation to avoid severe aggregation.

In fact, in eukaryotes there is considerable evidence for the idea that sHSPs play a role in facilitating protein degradation at several different points along the protein degradation pathway. For example, the mammalian sHSP Hsp27 facilitates the degradation of I-κBα, an inhibitor of the NF-κB transcription factor, by binding directly to ubiquitylated I-κBα and delivering it to the proteasome (Parcellier *et al.*, 2003). The sHSP αB-crystallin has been implicated in an earlier step in degradation, specifically, αB-crystallin interacts with FBX4 to form an SCF-family E3 ubiquitin ligase that specifically ubiquitylates cyclinD1, targeting it for degradation (den Engelsman *et al.*, 2003; Lin *et al.*, 2006). Finally, sHSPs have also been implicated in protein degradation via endoplasmic reticulum (ER)-associated degradation (ERAD). αA-crystallin specifically enhances the degradation of ΔF508-CFTR, a mutant version of the CFTR ion channel associated with cystic fibrosis, but not wild-type CFTR (Ahner *et al.*, 2007). Thus, in the case of mammalian sHSPs it certainly seems as though the sHSPs are able to influence the substrate choice of the proteasome at several different points in the proteasome-mediated degradation pathway.

Given the numerous examples for the involvement of eukaryotic sHSPs in targeting specific clients for degradation, it seems reasonable that the Ibps might also play a more active role in targeting particular substrates to Lon. The question of how the Ibps might affect Lon's substrate choice is a fascinating one. One way to answer this question would be to use the proteolytically inactive LonS679A mutant to trap Lon substrates in wild-type cells and in *Δibp* cells under different conditions. These

experiments would reveal whether there are any differences in the substrates that are trapped by Lon in the presence or absence of the Ibps, and may reveal if there are specific client proteins that the Ibps deliver to Lon. If such client proteins exist, it would be very interesting to explore these clients further. It would be particularly informative to determine what features these clients have in common. Are the sequences of these Ibp-dependent Lon substrates enriched in hydrophobic residues? Are they all involved in certain biological pathways (metabolism, cell division etc.)? The answers to these questions may give us insights into whole new areas of sHSP biology.

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