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Protein folding and degradation in bacteria: To degrade or not to degrade? That is the question

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Abstract. In *Escherichia coli* protein quality control is carried out by a protein network, comprising chaperones and proteases. Central to this network are two protein families, the AAA+ and the Hsp70 family. The major Hsp70 chaperone, DnaK, efficiently prevents protein aggregation and supports the refolding of damaged proteins. In a special case, DnaK, together with the assistance of the AAA+ protein ClpB, can also refold aggregated proteins. Other Hsp70 systems have more specialized functions in the cell, for instance HscA appears to be involved in the assembly of Fe/S proteins. In contrast to ClpB, many AAA+ proteins associate with a peptidase to form proteolytic machines which remove irreversibly damaged proteins from the cellular pool. The AAA+ component of these proteolytic machines drives protein degradation. They are required not only for recognition of the substrate but also for substrate unfolding and translocation into the proteolytic chamber. In many cases, specific adaptor proteins modify the substrate binding properties of AAA+ proteins. While chaperones and proteases do not appear to directly cooperate with each other, both systems appear to be necessary for proper functioning of the cell and can, at least in part, substitute for one another.

Key words. Chaperone; protease; folding; degradation; AAA+ superfamily; Hsp70; adaptor proteins.

Introduction

To maintain a competitive advantage over neighbouring populations, bacteria need to respond to different environmental challenges. For instance, following a sudden increase in temperature, the bacterial cell synthesizes a group of highly conserved heat-shock proteins (Hsps), many of which are commonly referred to as molecular chaperones. These proteins fall into two functionally distinct groups, chaperones and proteases. Together they form the main artillery for combating the effects of cellular stress, not only by directly removing damaged proteins but, in some cases, also coordinating the cellular response to stress by regulating the expression of chaperone and protease genes. How the cell coordinates such an attack on damaged proteins is currently of considerable interest. What determines the fate of an unfolded protein? Are there distinguishing features within an unfolded or damaged protein specific for refolding versus degradation and visa versa? Is there cooperation between chaperone and proteolytic systems, or do the two systems compete with one another for substrate binding? Although protein misfolding is often perceived to occur mainly during stress, many proteins also have difficulties to maintain their final folded state under normal cellular conditions. These difficulties in protein folding are often accentuated in large multidomain proteins. It has been suggested that the high protein concentration of the bacterial cytosol (340 mg/ml) [1] creates an unfavourable protein-folding environment that may lead to protein aggregation within the cell [2]. One physiologically important role of molecular chaperones is to prevent the accumulation of aggregated proteins and to promote the refolding of misfolded proteins (reviewed in [3, 4]). The function of the major chaperone systems in the *Escherichia coli* cytosol is summarized in table 1.

Generally, chaperones and proteases are thought to bind to exposed hydrophobic sequences, present in unfolded

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Table 1. Major chaperones and proteases of the *E. coli* cytosol.

Name*	Family	Function	Phenotype of gene deletion
Chaperones			
GroEL	Hsp60	Protein folding (with GroES),	lethal
DnaK	Hsp70	Protein folding (with DnaJ/GrpE), gene regulation, DNA replication	lethal >37 °C and <20 °C, stress sensitive
HscA (Hsc66)	Hsp70	Fe/S assembly (with HscB)	Slow growth
HscC	Hsp70	?	No phenotype
Trigger factor	PPIase	Prevention of aggregation of newly synthesized proteins	No phenotype, but essential in Δ*dnaK*
HtpG	Hsp90	?	Reduced growth rate at 44 °C
ClpB	AAA+	Protein disaggregation, (with DnaK/DnaJ/GrpE)	Impaired thermotolerance
ClpA (Ti)	AAA+	Protein degradation (with ClpP)	No phenotype
ClpX	AAA+	Protein degradation (with ClpP)	No phenotype
HslU (ClpY)	AAA+	Protein degradation (with HslV)	No phenotype
Hsp33	Hsp33	Redox regulated	Slight sensitivity towards H_2O_2 and high temperature
IbpA/IbpB	Small Hsps	Prevention of protein aggregation	No phenotype
Proteases			
FtsH (HflB)[a]	AAA+	Protein degradation	lethal
Lon	AAA+	Protein degradation	Mucoid growth
EfcE[a]		Protein degradation	lethal
ClpP	peptidase	Protein degradation (with ClpA or ClpX)	No phenotype
HslV (ClpQ)	peptidase	Protein degradation (with HslU)	No phenotype

* Alternative names are written in brackets.
[a] Membrane associated protease.

or misfolded proteins. In some cases, however, specific recognition signals, either intrinsic or added (e.g. SsrA-tagged proteins), may also play an important role in targeting a substrate to a particular chaperone or protease. In bacteria the SsrA-tagging system has been developed to rid the cell of incompletely synthesized proteins. These 'nonsense' proteins, which result from stalled synthesis on the ribosome, are tagged for destruction by the co-translational addition of an 11-amino acid peptide (AANDENYALAA) to the C-terminus by an extraordinary molecule known as tm-RNA (reviewed in [5]). This process serves not only to facilitate the degradation of incomplete proteins, but also to recycle stalled ribosomes for further rounds of protein synthesis. The resulting SsrA-tagged proteins can be degraded, in vitro, by several different ATP-dependent proteases, including ClpXP, ClpAP and FtsH.

Chaperones and proteases are involved not only in general protein quality control but also in regulation and in the management of specific protein-folding pathways. In this review we will first examine the general role of chaperones and proteases in protein quality control and the interplay between these two systems, and finally examine the more specialized role of an Hsp70 chaperone system in the assembly of Fe/S cluster proteins. For the sake of clarity, we have divided the molecular chaperones into three distinct groups according to their mode of action: holders, folders and unfolders. Folders (e.g. DnaK and GroEL) refer to a class of molecular chaperones, which probably through substrate unfolding (either local or global) result in the refolding of misfolded or aggregated substrates, while holders refer specifically to proteins that can prevent protein aggregation but are themselves unable to refold the substrate (e.g. small Hsps). Finally, since the primary role of many Clp/Hsp100 proteins is not to refold protein substrates but rather to unfold them in preparation for their subsequent degradation (by an associated peptidase) or refolding (by a folder cochaperone) we have used the term 'unfolders' to refer specifically to Clp/Hsp100 chaperone-like proteins (e.g. ClpA and ClpB). These definitions, however, are not intended to suggest that folders are not unfolders; on the contrary it has been shown that GroEL mediates protein folding through partial unfolding of its substrate [6]. Furthermore, we speculate that DnaK may also drive protein folding by unfolding its substrate. Therefore, here we define folder chaperones not by their ability to unfold, but rather by their ability to refold misfolded proteins.

Bacterial AAA+ chaperones (unfolders)

Clp/Hsp100 proteins can be classified into two distinct subfamilies [7]. Class I proteins (e.g. ClpA and ClpB in *E. coli* and other Gram-negative bacteria; ClpC and ClpE in *Bacillus subtilus* and other Gram-positive bacteria) are composed of an N-terminal variable region and two highly conserved nucleotide binding domains (NBDs), D1 and D2 (fig. 1A). Both NBDs contain typical Walker A and Walker B motifs required for ATP binding and hydrolysis, respectively. In contrast, class II proteins (e.g. ClpX and HslU) contain only a single NBD (homologous to D2), and in the case of ClpX a Zn binding domain at the N-terminus. Interestingly, HslU does not contain a N-terminal domain but rather contains an extra domain (the I domain), inserted into the D2 domain. Members of both protein classes form ring-shaped oligomers (generally hexamers) upon ATP binding. The most striking difference within members of the class I subfamily is the presence or absence of a region that links the two NBDs, D1 and D2. This region, often referred to as the linker, is proposed to form a coiled coil which is repeated four times in ClpB and twice in ClpC. Its function is poorly studied, although as a coiled coil, it is likely to play a role in protein-protein interactions, either in protein oligomerization or perhaps in substrate binding.

Recently Neuwald and colleagues [8] showed, using a combination of database searching and multiple sequence alignment, that AAA (ATPase associated with a variety of cellular activities) and Clp/Hsp100 proteins share considerable sequence homology. The regions of homology were generally confined to structurally important parts of the molecule required for ATP hydrolysis and protein oligomerization. Consequently, a new superfamily (the AAA+ superfamily), comprising Clp/Hsp100 and AAA proteins was proposed [8]. The structural basis of this superfamily was later confirmed following the determination of the first Clp/Hsp100 protein structure, HslU [9, 10] (see fig. 1B), which showed significant similarity to the AAA protein, *N*-ethylmaleimide-sensitive fusion protein (NSF) and p97 [11, 12]. Although as the name suggests, AAA+ proteins are involved in a variety of different cellular functions, this review will concentrate only on a subset of AAA+ proteins involved in protein quality control. In some cases AAA+ proteins (e.g. Lon and FtsH) contain two functionally distinct modules within the same polypeptide, a chaperone-like AAA+ module and a peptidase module.

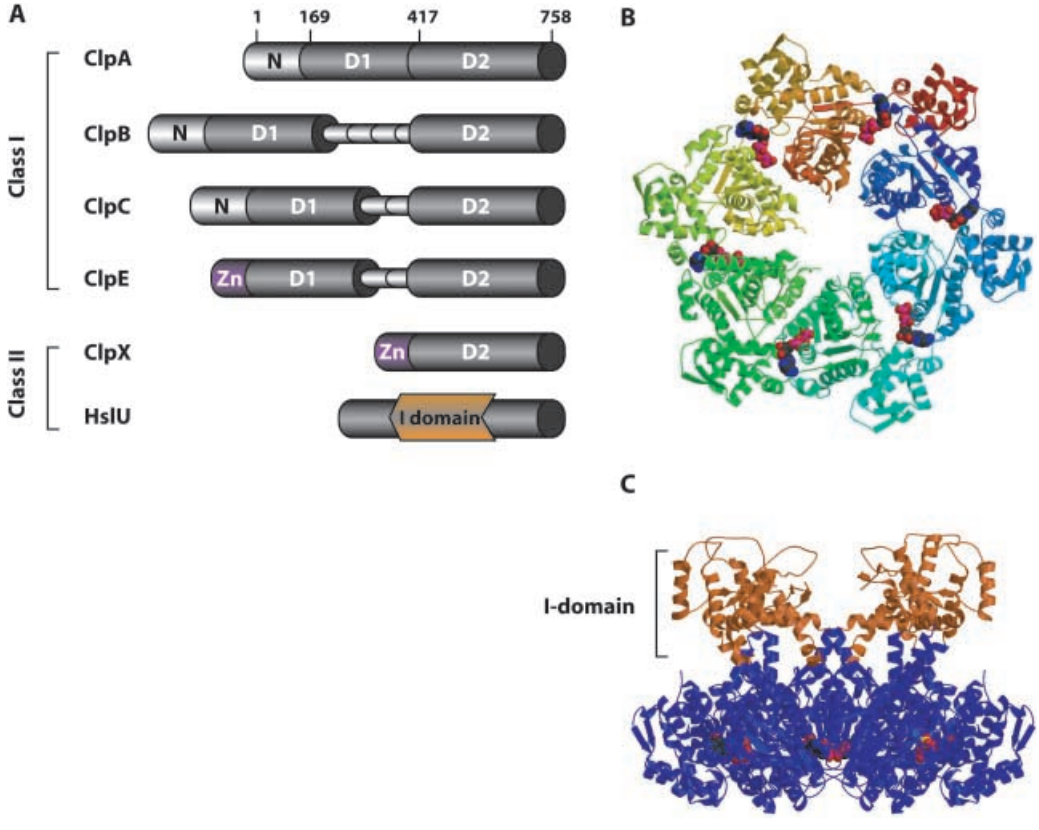

Figure 1. (*A*) Domain structure of bacterial AAA+ proteins. Class I proteins contain a variable N-terminal domain and two NBDs separated by a linker domain of variable length. Class II proteins contain only one NBD. (*B*) The hexameric, ring-shaped structure of HslU is conserved in all AAA+ proteins. (*C*) The I domain (in brown) of HslU is located at the distal face of the HslUV complex, poised to feed substrates through the cavity of HslU and into the proteolytic chamber of HslV.

However, in the majority of cases, many AAA+ proteins participate in proteolysis although they do not contain a peptidase module (i.e. ClpA, ClpC, ClpX and HslU). These AAA+ proteins regulate protein degradation by association with a ring-shaped peptidase [9, 13]. For instance, ClpA, ClpX and ClpC all interact with the peptidase ClpP, while HslU regulates HslV-mediated degradation. Recently, an essential ClpP docking site in ClpX was identified [7]. The site consists of a tripeptide ([LIV]-G-[FL]), located within the D2 domain, which is also conserved in the other ClpP-interacting proteins, ClpA and ClpC. Mutation of hydrophobic residues in this tripeptide prevented the ClpP-dependent degradation of certain ClpX-mediated substrates, but not the ClpP-independent chaperone activity of ClpX [14]. Interestingly, this region is absent in all ClpB homologs examined, thereby explaining why ClpB acts independent of the peptidase ClpP despite the large overall similarity to ClpA. Rather, ClpB functions together with the DnaK chaperone system to disaggregate and refold aggregated proteins within the cell (see later).

Substrate interaction

Currently there is considerable debate regarding the number and location of the substrate binding sites in AAA+ proteins. Is there more than one substrate binding site within each subunit of an AAA+ protein? If there are multiple binding sites, do these different binding sites interact with distinct types of substrates? Unfortunately the structural information available is limited, and consequently it has been difficult to identify potential binding sites within AAA+ proteins. Interestingly, the unfoldase HslU contains a discrete domain (the I domain) located at the distal end of HslU within the HslUV complex (fig. 1 C). This places the I domain in an ideal position to feed substrates, through HslU, into the proteolytic chamber of HslV. Deletion of this domain blocks the HslV-mediated degradation of some HslU-dependent substrates, and hence has been implicated in substrate binding. Nevertheless the degradation of casein was not affected by deletion of the I domain, suggesting at least two possibilities: either HslU contains multiple substrate binding sites or, alternatively, some substrates exhibit different requirements for unfolding. Unfortunately, however, this domain is unique to HslU and not found in other AAA+ proteins. To identify the substrate binding of other AAA+ proteins alternative approaches have been taken. Initially, it was shown, using ELISA (enzyme-linked immunosorbent assay) that the isolated C-terminal fragment of several different AAA+ proteins could interact alone with appropriate substrates [15]. This region was therefore termed the sensor- and substrate-discrimination (SSD) domain. Similarly, the C-terminal region of *E. coli* ClpB was shown to be essential for luciferase refolding [16].

Several pieces of evidence, however, suggest that the C-terminal regions of the AAA+ proteins tested are unlikely to interact directly with substrates; rather, they appear to be essential for oligomerization. First and most importantly, analysis of the HslU structure shows that the SSD domain is indeed part of the second NBD and not a separate domain [9, 10]. This region makes critical contacts between adjacent monomers within the hexamer and thereby mediates oligomer formation. Consistent with this role for the SSD, mutants of ClpB lacking the SSD failed to oligomerize [16]. Second, the experimentally determined tripeptide identified in ClpX, involved in peptidase binding, also lies in close proximity to the SSD, suggesting that substrate degradation would require the unlikely binding of both the substrate and the peptidase to the same region of the unfoldase. This model also places the N-terminal domain at the distal surface of ClpA or ClpX in the ClpAP and ClpXP complexes, and hence the N domain is more likely to contact the substrate. Recently, using electron microscopy, Stevens and colleagues [17, 18] were able to show that this was indeed the case for the ClpX and ClpA proteins. Using λO and RepA as substrates, they identified extra density bound to the distal end of ClpX and ClpA, respectively. Finally, several groups have shown that the N-terminal domain of both ClpA and ClpB played an important role in the recognition of some substrates [16, 19, 20]. Deletion of the N-terminal domain of ClpA modified the degradation of SsrA-tagged GFP dramatically and reduced the rate of λ N-cI-SsrA degradation, although it had little effect on the degradation of casein [19]. Similarly, the N domain of ClpB was required for the reactivation of unfolded luciferase [16]; surprisingly, however, deletion of the N-terminal domain from ClpB does not affect its in vivo activity (e.g. development of thermotolerance) in *E. coli* [A. Mogk, unpublished]. Therefore, it would seem that the N-terminal domain is not required for all substrates; rather it may only be required for specific substrates.

Consistent with this view, the *E. coli* genes encoding ClpA and ClpB contain internal initiation sites from which N-terminally truncated versions of these proteins are produced in vivo. Interestingly, the cyanobacterium *Synechococcus* sp. strain PCC 7942 contains two ClpB homologs, one classical ClpB containing an internal start codon, involved in thermotolerance, and a second, essential, ClpB of unknown function (termed ClpBII). While the N domain of the classical ClpB is dispensable for activity, the N domain of ClpBII is essential for protein function [21]. This suggests that in the case of ClpBII, the N-terminal domain may form a specialized binding site for a substrate. Finally, regardless of the number of different binding sites that may exist within a molecule, what remains unclear is whether more than one binding site (of the same type) is required to efficiently bind and subsequently unfold the substrate. Probably, in the case of

N- or C-terminal substrate tags such as SsrA, the initial binding only requires a single binding site. However, it remains a challenge for the future to determine whether a stable interaction between other more general substrate recognition motifs, such as hydrophobic patches, only occurs in the presence of multiple low-affinity interactions between the substrate and the oligomeric form of the unfoldase.

AAA+ adaptor proteins

Substrate recognition by AAA+ proteins is not always a direct reflection of the binding activity of the AAA+ protein itself; the recognition may in some cases be modified by the use of adaptor proteins. Although unrelated both in sequence and structure [22], adaptor proteins can be grouped together based on their ability to modify the properties of an AAA+ protein. In general, adaptor proteins assert their effects on their AAA+ partner protein through substrate binding. Most adaptor proteins interact directly with the substrate, possibly mediating the transfer of the substrate to the AAA+ protein. In some cases, however, it appears that the adaptor protein alone does not bind the substrate. Instead, it only interacts with the substrate in complex with the AAA+ partner protein. In bacteria, a handful of such adaptor proteins have been identified. The first such protein, MecA, is essential for the ClpCP-mediated degradation of regulatory proteins involved in the development of competence (ComK and ComS) in *B. subtilis* [23]. However its involvement in more general ClpCP-mediated activities is still unknown. In *E. coli* several different adaptor proteins have been characterized. Often they are only required for the degradation of specific substrates. For instance, in the degradation of SsrA-tagged proteins, both ClpXP- and ClpAP-mediated degradation can be specifically modulated by different adaptor proteins. The ClpXP machine uses the ribosome-associated adaptor protein SspB, which binds directly to the SsrA-tag [24], to specifically enhance the ClpXP-mediated degradation of SsrA-tagged proteins. Similarly, RssB, a two-component response regulator, mediates its effect through binding to the ClpXP substrate, the starvation sigma factor, σ^S [25, 26]. Consequently both adaptor proteins only modulate recognition of specific substrates and do not affect the degradation of other ClpXP substrates. Recently, a ClpA-specific adaptor protein (ClpS) was identified in *E. coli* [20]. The gene encoding ClpS is located in an operon together with *clpA*. ClpS, a unique adaptor protein, was shown to regulate the degradation of two different ClpAP-mediated substrates. Recognition (by ClpA) and consequently degradation (by ClpAP) of aggregated proteins was enhanced by ClpS. In contrast, ClpS was shown to inhibit both binding and degradation of SsrA-tagged substrates. Therefore, ClpS redirects ClpAP activity away from SsrA-tagged proteins and potentially towards aggregated proteins. This ClpS-mediated switch in ClpA substrate specificity is regulated by interaction of ClpS with the N-terminal domain of ClpA and not through interaction with the substrate. Furthermore, ClpS binding to the N domain also triggers the release of prebound SsrA-tagged substrates, suggesting that binding of ClpS may induce a conformational change in ClpA. Interestingly, the role of these adaptor proteins finally resolves long-standing inconsistencies between the in vitro and in vivo effectiveness of both proteolytic machines, ClpAP and ClpXP, towards SsrA-tagged proteins. ClpS blocks the binding of SsrA-tagged proteins to ClpA, while SspB, located at the ribosome, efficiently redirects SsrA-tagged proteins to the ClpXP system. Therefore, in vivo, ClpXP is the major protease responsible for degradation of SsrA-tagged proteins [27].

Protein degradation

Substrate unfolding and translocation

ClpA has proven to be an important model for studying mechanistic aspects of AAA+ (Clp/Hsp100) proteins not only as an ATP-dependent unfoldase but also as the regulatory component of the ClpAP proteolytic machine (reviewed in [28, 29]). Protein degradation is a three-step process; (i) substrate recognition, (ii) substrate unfolding and (iii) translocation into the active chamber of the peptidase. Although little progress has been made in the area of substrate recognition by the chaperone component, considerable advances have been made in the areas of substrate unfolding and translocation into the proteolytic chamber. Since the initial demonstration of a 'chaperone-like' activity of ClpA using firefly luciferase as a model substrate [30], much of our understanding of ClpA activity has stemmed from the analysis of two different model substrates, the plasmid replication factor RepA, and SsrA-tagged GFP. The use of GFP-SsrA has been instrumental in demonstrating many ClpA- and ClpX-related activities. Recently, a series of elegant experiments showed that ClpA mediates the global unfolding of protein substrates [31]. By monitoring the fluorescence of GFP-SsrA in the presence of ClpA and a GroEL-Trap (D87K), which binds but does not release unfolded proteins, Horwich and colleagues [31] showed that ClpA is able to unfold GFP-SsrA. Furthermore, by comparing the hydrogen-deuterium exchange of GFP-SsrA in the presence and absence of ClpA, they were able to determine that ClpA was responsible for the complete unfolding of the substrate. Subsequently, similar experiments were used to demonstrate the unfolding activity of ClpX [32].

Using a combination of cryo- and negatively stained electron microscopy, Stevens and colleagues examined the translocation pathways of ClpXP [17] and ClpAP [18] by following the fate of λO and RepA, respectively. They were

able to visualize several discrete steps of substrate translocation from the initial binding of the substrate to final release of the degraded peptides (fig. 2). In general, the principles of protein translocation did not change for the different proteolytic machines tested (ClpAP and ClpXP). Initial binding of the substrate occured at the distal region of the proteolytic machine, thereby providing further evidence that the N-terminal region is important for substrate binding. Using two different forms of the catalytically inactive ClpP [one which contains the pro-peptides and blocks entry into the catalytic chamber ($ClpP_{SC}$) and the other in which the catalytic residue is chemically inactivated ($ClpP_{in}$)], the authors were able to trap the substrate at different points during the translocation. In the presence of $ClpP_{SC}$ the substrate accumulates first at the inner surface of the distal ATPase and, to a lesser extent, at the inner surface of the proximal ATPase, while in the presence of $ClpP_{in}$ the bulk of the substrate was identified in the catalytic chamber of ClpP. Following degradation by the active peptidase, the degraded peptides are released into the solution surrounding the peptidase [18]. The directionality

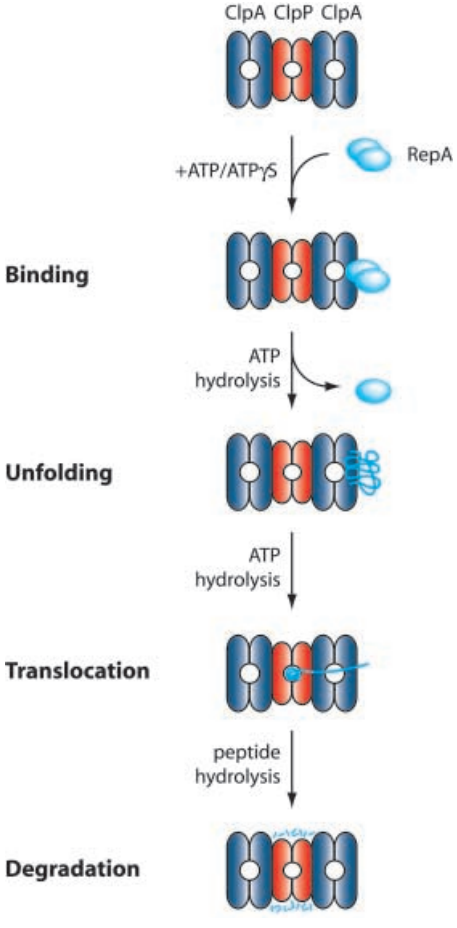

Figure 2. ATP-dependent proteolysis. Schematic model illustrating the steps of protein degradation by the ClpAP machine based on the model ClpA-substrate RepA. These steps have also been elucidated for the degradation of λO by the ClpXP machine.

of substrate translocation, through ClpA, into the ClpP chamber was elucidated using a combination of time-dependent fluorescent anisotropy and fluorescence resonance energy transfer (FRET). By labelling ClpP with a donor fluorophore and different substrates with an acceptor fluorophore, it could be demonstrated that the C-terminus of SsrA-tagged substrates (where the tag is located) was translocated into the ClpP chamber before the N-terminus [33]. This suggests that SsrA-tagged substrates are threaded in a linear fashion through a narrow passage in ClpA into the catalytic chamber of ClpP.

Protein disaggregation

In contrast to other AAA+ proteins, ClpB is not involved in proteolysis but rather acts in collaboration with the DnaK system to disassemble and refold large protein aggregates [34–37]. The disaggregating activity of this bichaperone system becomes especially important at very high temperatures. Under these conditions, strong protein aggregation occurs because the limited holder chaperone capacity of the cell cannot suppress the large increase in misfolded proteins. The survival of cells subjected to severe heat stress (development of thermotolerance) relies on reactivation of these aggregated proteins. Analysis of *dnaK* and *clpB* null mutant strains showed that protein disaggregation in vivo and development of thermotolerance is strictly dependent on DnaK and ClpB [35, 38, 39]. Increased expression of *dnaK*, *dnaJ*, *grpE* and *clpB* is necessary and sufficient to restore thermotolerance in *ΔrpoH* mutant cells, which lack the transcriptional activator of the heat shock regulon, σ^{32}, and therefore exhibit strongly reduced chaperone and protease levels [40]. In agreement with these findings, knockout mutations of *hsp104*, the eukaryotic ClpB homolog, in *Saccharomyces cerevisiae* and *Arabidopsis thaliana* also prevent cells from acquiring thermotolerance and surviving severe stress [41, 42]. Therefore, following heat stress, the ability of these cells to reverse protein aggregation, not only in eubacteria but also in yeast and plants, is linked to thermotolerance and survival.

The refolding of aggregated proteins by the bichaperone machinery could be separated into two phases using inorganic sulfate as a specific inhibitor of ClpB activity. First, aggregated proteins are resolubilized by ClpB and DnaK, while the subsequent refolding of the disaggregated substrates needs only DnaK [34]. Currently, the precise mechanism of DnaK/ClpB cooperation during protein disaggregation is unknown; however there are two working models. The first model favours sequential action of both chaperones. ClpB is proposed to interact first with protein aggregates, thereby changing aggregate structure and increasing substrate accessibility to DnaK. This model is supported by the finding that substoichiometric amounts of ClpB in relation to DnaK are sufficient to allow fast re-

folding of the aggregated model protein malate dehydrogenase [34]. In addition, a disaggregating activity of DnaK towards small protein aggregates has been demonstrated [43]. The second model suggests that resolubilization of protein aggregates is achieved by a ClpB/DnaK complex and is based on the observation that cooperation of the two chaperone systems, Hsp70 and Hsp100, exists only between proteins of the same species [44].

Interplay between chaperones and proteases

Misfolded or aggregated proteins expose hydrophobic patches at their surface which provide the interaction sites for chaperones and proteases for their subsequent refolding or degradation. The functional interplay between proteases and chaperones in this process is poorly understood. The protein quality control network is depicted in figure 3. Do chaperones and proteases compete for a common substrate pool, or do chaperones assist protein degradation? Can proteases substitute for missing chaperone function in vivo (and vice versa)?

Mutations in genes encoding major chaperones (DnaK/DnaJ/GrpE and GroEL/GroES) lower the efficiency of degradation of misfolded proteins in the *E. coli* cytosol [45–47]. This led to the suggestion that chaperones participate directly in protein degradation in vivo, and it has even been speculated that this occurs via handing over of substrates to the proteolytic machinery through physical association between chaperones and proteases [48]. However, many protein variants used in these studies failed to reach the native conformation, creating an artificial situation. Beneficial effects of chaperones on protein degradation can alternatively be explained by stabilizing soluble forms of nonnative protein substrates through cycles of binding and release [28, 49]. The fate of a substrate, whether it will be refolded by chaperones or, in a competing reaction, be degraded by proteases may simply be determined by the binding affinities (kinetic partitioning) of the corresponding chaperones and proteases [50]. A final possibility suggests that chaperones such as GroEL and perhaps DnaK, through their unfolding activity, present substrate in conformations which are recognized by proteolytic systems.

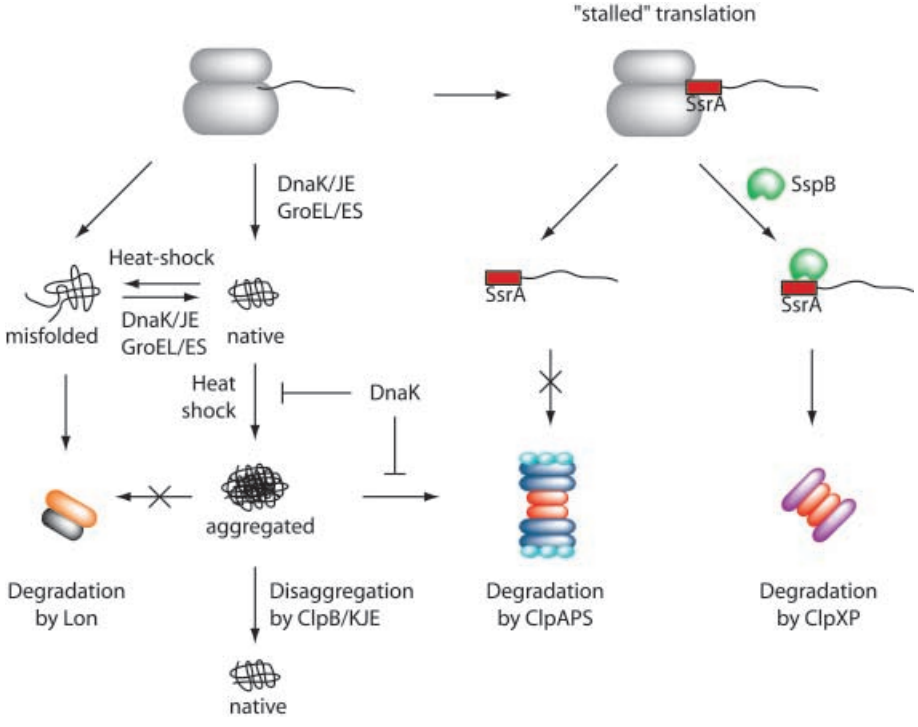

Figure 3. Schematic illustration of protein quality control in *E. coli*. Most proteins synthesized by the ribosome fold to their native state with the assistance of either the DnaK or GroEL chaperone systems (KJE or ELS, respectively). Following heat shock some proteins may misfold, as may some newly synthesized proteins. The misfolded proteins may be refolded to the native state by the KJE or ELS systems, or alternatively they are degraded by the protease Lon. Under severe conditions of stress, however, in the absence of sufficient DnaK levels, these misfolded proteins tend to aggregate. The aggregated proteins may either be rescued by the ClpB/KJE bi-chaperone system or degraded by the ClpAPS proteolytic machine; however, the refolding of aggregated proteins is favoured over their degradation. In some cases protein synthesis may stall at the ribosome; these proteins are specifically tagged for destruction. The tag (SsrA) addresses the unfinished proteins to the proteolytic machines ClpAP and ClpXP. Degradation of tagged proteins by the machines is modulated by two adaptor proteins. ClpS specifically inhibits ClpAP-mediated degradation of SsrA-tagged proteins, while SspB enhances the degradation by ClpXP. Consequently, in the cell the ClpXP-mediated degradation of these tagged proteins is favoured.

Synergistic and overlapping functions of proteases and chaperones in the protein quality control system have been recently demonstrated. Lon, ClpAP, ClpXP and HslUV, representing the major ATP-dependent proteolytic systems of the *E. coli* cytosol, act synergistically in vivo in the degradation of abnormal proteins. *E. coli* cells lacking several but not the individual proteases are unable to grow at high temperatures around 45 °C [51]. Interestingly, already at 42 °C the proteases ClpXP and Lon become essential if the levels of the DnaK chaperone are reduced. Furthermore, under normal growth conditions at 30 °C and especially at high temperatures, depletion of the DnaK system already causes strong protein aggregation both in *clpXP* and *lon* mutant cells, but not in wild-type cells, indicating a synergistic affect of proteases and the DnaK system in preventing protein aggregation [40]. These findings also suggest that efficient degradation of misfolded proteins is not strictly dependent on chaperone activity, and supports an indirect role of chaperones in protein degradation.

The DnaK/Hsp70 Family

Currently, many extensive reviews describing all aspects of DnaK are available [4, 52], while none have yet focused on the remaining members of the Hsp70 family in *E. coli*, HscA (Hsc66) and HscC (Hsc62). Therefore, in this review, we chose to place greater importance on these alternative Hsp70 chaperone systems with a particular emphasis on the HscA system.

In general, all Hsp70 proteins appear to share a common structure. They comprise two domains, a N-terminal ATP binding domain and a C-terminal substrate binding domain. For *E. coli* DnaK it was shown that binding and hydrolysis of ATP modulates the affinity of substrate to the C-terminal domain. When ADP is bound, DnaK is able to bind substrates in a stable manner and the substrate is only released when ATP is bound. To efficiently regulate this cycle of substrate binding and release, DnaK cooperates with several different partner proteins. The J proteins (DnaJ, CbpA and DjlA), which share a conserved J domain, interact with the ATPase domain of DnaK, thereby coupling ATP hydrolysis with substrate binding, while in the final step of the refolding cycle, DnaK utilizes a unique nucleotide exchange factor, GrpE, to accelerate the release of ADP from its ATPase domain [53]. Thus J proteins stimulate substrate binding to DnaK, while GrpE enhances substrate release by accelerating nucleotide exchange of DnaK. As the most abundant chaperone in bacteria, DnaK plays a major role in stress management. It is involved both directly through the efficient binding to unfolded protein substrates and indirectly through regulation of other chaperone proteins via the stress-dependent association with the heat shock transcription factor σ^{32}.

The other bacterial Hsp70 proteins appear to cooperate only with specific J proteins and hence are probably required to deliver specific substrates to their Hsp70 partner chaperones. Currently, our knowledge of HscC is extremely limited [54]. Deletion of *hscC* has no known phenotype, and overproduced HscC is unable to complement a *ΔdnaK* strain. Therefore, it seems likely that HscC may be involved in a specialized chaperone function. HscC is proposed to function together with one of two putative J proteins (YbeV and/or YbeS), the genes of which are located in the vicinity of *hscC* [55]. Currently, however, there is no evidence for the existence of such an interaction, and furthermore a substrate for this putative chaperone system has yet to be identified.

HscA: A novel Hsp70 system for a specific folding problem

HscA is constitutively expressed in *E. coli*. The *hscA* gene is cotranscribed with an upstream *dnaJ*-like gene (*hscB*); however, no *grpE*-like gene was found. Together, HscA and HscB (Hsc20) form a unique chaperone system. Since nucleotide exchange normally plays a crucial role in substrate release, it was intriguing that this system did not utilize a nucleotide exchange factor. A detailed analysis of HscA revealed that a number of subtle variations in the ATP binding domain of HscA were responsible for large changes in the properties of ATP binding and hydrolysis [56, 57]. HscA proteins of eubacteria form a subfamily of Hsp70 proteins in which a small loop, termed the GrpE signature loop, and two salt bridges in the ATPase domain interface are absent. HscA utilizes these unique properties, to regulate substrate binding and release, in the absence of a nucleotide exchange factor like GrpE. When compared with other Hsp70s, HscA has a low affinity for ATP while the exchange of ADP is relatively high, hence a nucleotide exchange factor is not needed.

In the search for substrates which may require HscA, the first hint came from work carried out in *Saccharomyces cerevisiae*. Culotta and colleagues [58] identified *SSQ1*, *JAC1* and *NFS1*, homologs of *hscA*, *hscB* and *iscS*, respectively, as suppressor genes in *sod1Δ* mutants (mutations in the *E. coli sod* gene were implicated in the inactivation of some Fe/S proteins). Interestingly in *E. coli*, *iscS* was found in an operon together with two other genes (*iscU* and *iscA*), and these genes were located in a gene cluster together with the *hscAB* operon, which led to the speculation that IscU, a Fe/S scaffold protein, may require the HscA/HscB chaperone system for Fe/S assembly. Initially it was shown that both IscU and the IscU-Fe/S complex could interact with HscA, in an HscB-dependent fashion [59]. The ATPase activity of HscA was stimulated by both forms of the substrate, with maximal stimulation achieved in the presence of the co-chaperone HscB [59]. Typical for many J proteins, HscB was demonstrated to

form a complex with the substrate (IcsU), which presumably aids in targeting the substrate to HscA, since binding of HscA to IscU was enhanced in the presence of HscB. Recently, it was demonstrated that IscU inhibits the general chaperone activity of HscA and furthermore interacts specifically with the substrate binding domain of HscA [60]. This suggests a role for the HscA chaperone system in Fe/S assembly of IscU, which may in turn serve as a scaffold for the assembly of other Fe/S proteins. Consistent with these findings, it was reported that overproduction of the ORF1-ORF2-*iscSUA-HscBA-fdx*-ORF3 gene cluster facilitated the assembly of Fe/S clusters into a number of Fe/S proteins [61]. Furthermore, the genetic dissection of this cluster confirmed that both HscA and HscB were essential for the assembly of several Fe/S proteins in vivo [62].

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