Chaperone-Assisted Protein Folding in the Cell Cytoplasm

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Abstract: Folding of polypeptides in the cell typically requires the assistance of a set of proteins termed molecular chaperones. Chaperones are an essential group of proteins necessary for cell viability under both normal and stress conditions. There are several chaperone systems which carry out a multitude of functions all aimed towards insuring the proper folding of target proteins. Chaperones can assist in the efficient folding of newly-translated proteins as these proteins are being synthesized on the ribosome and can maintain pre-existing proteins in a stable conformation. Chaperones can also promote the disaggregation of preformed protein aggregates. Many of the identified chaperones are also heat shock proteins. The general mechanism by which chaperones carry out their function usually involves multiple rounds of regulated binding and release of an unstable conformer of target polypeptides. The four main chaperone systems in the *Eschericia coli* cytoplasm are as follows. (1) Ribosome-associated trigger factor that assists in the folding of newly-synthesized nascent chains. (2) The Hsp 70 system consisting of DnaK (Hsp 70), its cofactor DnaJ (Hsp 40), and the nucleotide exchange factor GrpE. This system recognizes polypeptide chains in an extended conformation. (3) The Hsp 60 system, consisting of GroEL (Hsp 60) and its cofactor GroES (Hsp 10), which assists in the folding of compact folding intermediates that expose hydrophobic surfaces. (4) The Clp ATPases which are typically members of the Hsp100 family of heat shock proteins. These ATPases can unfold proteins and disaggregate preformed protein aggregates to target them for degradation. Several advances have recently been made in characterizing the structure and function of all of these chaperone systems. These advances have provided us with a better understanding of the protein folding process in the cell.

INTRODUCTION

The amino acid sequence of a given protein pre-determines the native state as well as the folding pathway of that protein [1-2]. However, since the environment in the cell is very crowded [3-4] and viscous [5-7], there is high probability that newly-translated proteins will misfold and aggregate. Since the successful folding of newly-translated proteins is essential to the viability of the cell, quality control mechanisms are present in the cell cytoplasm to insure that the folding process of any newly-synthesized polypeptide chain results in the formation of properly folded protein and that the folded protein is maintained in an active conformation throughout its functional lifetime [8-9]. Molecular chaperones are key components of this quality control machinery. They are a fundamental group of proteins that have been identified only relatively recently [10]. They have been shown to play essential roles in cell viability under both normal and stress conditions by assisting in the folding of newly translated proteins and by maintaining the conformational integrity of pre-existing proteins [11]. Chaperones can also assist in the unfolding of misfolded proteins and in disaggregating preformed protein aggregates [12]. Most molecular chaperones are also induced by heat shock – i.e. they are heat shock proteins.

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An operational definition of molecular chaperones in the context of protein folding can be given as follows [13]: a molecular chaperone is a protein that binds to and stabilizes an unstable conformer of another polypeptide and, through regulated binding and release cycles, facilitates the correct folding or assembly of the substrate polypeptide. The term "molecular chaperone" itself was first used by Laskey et al. [14] to describe nucleoplasmin, an acidic nuclear protein required for the assembly of nucleosomes from DNA and histones in extracts of eggs of the toad *Xenopus.* The term was then generalized by John Ellis [15].

A newly-synthesized nascent chain needs to be protected against unproductive interactions in at least four stages of its folding process: (1) while still attached to the ribosome as a nascent chain, (2) after release from the ribosome but while still in an extended conformation, (3) as a compact folding intermediate with exposed hydrophobic surfaces that eventually should be buried in the native state, and (4), in case of aggregation, as a misfolded protein. Both prokaryotes and eukaryotes have similar general chaperone systems which assist in the proper folding of polypeptide chains at these four different stages.

Different chaperone systems seem to differentiate between the different target substrates either based on the conformation or the sequence of the protein substrate. Molecular chaperones are known to be very promiscuous *in vitro* [16-17]. In a test tube, they have the ability to bind to a wide range of proteins in several conformational states. However, *in vivo*, there is evidence that chaperones have high

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Table 1. Four Main Chaperone Systems in *E. coli*

Chaperone System	Chaperones and Cofactors	SwissProt ID	Molcular Weight, KDa
TF	Trigger factor	TIG_ECOLI	48.2
Hsp 70	DnaK (Hsp 70) DnaJ (Hsp 40) GrpE	DNAK_ECOLI DNAJ_ECOLI GRPE_ECOLI	69.0 41.0 21.8
Hsp 60	GroEL (Hsp 60) GroES (Hsp 10)	CH60-ECOLI CH10_ECOLI	57.1 10.4
Clp ATPases	Clp (Hsp 100, class I)‡ ClpB (Hsp 100, Class I)† ClpX (Hsp 100, class II) ClpY (Hsp 100, class II)	CLPB_ECOLI CLPA_ECOLI HSLU_ECOLI CLPX_ECOLI	95.6 84.2 49.6 46.2

affinity for a defined set of proteins, and, therefore, preferentially assist in the folding and conformational maintenance of only this set of proteins.

Here we discuss our current understanding of the structural and functional properties of the bacterial chaperones. The discussion will focus on the following four main chaperone systems in *Eschericia coli*: trigger factor, the Hsp 70 system (DnaK/DnaJ/GrpE), the Hsp 60 system (GroEL/GroES), and the Clp ATPases (ClpA/ClpB/ ClpX/ClpY). Chaperones and cofactors belonging to these four systems are listed in (Table **1**). Close homologues of these chaperones are present in all kingdoms of life [18].

TRIGGER FACTOR:

Assisting the Folding of Nascent Chains

Folding of a newly-translated protein can occur while the polypeptide chain is still attached to the ribosome. The binding of chaperones to nascent chains seems to be essential for proper protein folding. Chaperones, at this early stage in the life of a protein, maintain nascent chains in a conformation competent for folding upon subsequent release and function to protect nascent chains from unproductive interactions with the surface of the ribosome or with other nascent chains and factors in the cytoplasm. Consequently, it has been observed that cell viability critically depends on the presence of ribosome-associated chaperones. Trigger factor, and to some extent DnaK (see next section), are two such chaperones.

Trigger factor was initially discovered in *E. coli* as a cytosolic protein which maintains a secretory precursor outer membrane polypeptide (proOmpA) in a loosely folded conformation competent for membrane translocation [19-21]. Subsequent studies, however, in which the cellular content of trigger factor was reduced, revealed no secretion defect of proOmpA [22]. Subsequently, it was established that trigger factor is a ribosome-bound factor which can be efficiently cross-linked to secretory and non-secretory polypeptide chains [23] only when these chains are bound to ribosomes [24]. For non-secretory substrates, trigger factor was the single major cross-linking component of the *E. coli* cytoplasm [23].

The binding of trigger factor to ribosomes is specifically to the large ribosomal subunit which contains the exit site for the nascent chains. It is reasonable to speculate that trigger factor binds next to the exit site on the ribosome for efficient association with newly-translated substrate nascent chains. In this regard, there seems to be some overlap in the function of trigger factor with that of DnaK which also binds to nascent chains (see below). *E. coli* strains which are deleted of trigger factor show no defects under normal growth conditions, but when trigger factor deletion is combined with the deletion of the gene for the general chaperone DnaK, then the cells are not viable at 37^{o}C [25-26]. These observations demonstrate that chaperone binding to nascent chains is essential for cell viability.

In vitro, trigger factor was found to exhibit very strong peptidyl-prolyl cis/trans isomerase activity (PPIase) [27] and to efficiently catalyze protein folding reactions which are rate-limited by the isomerization of prolyl peptide bonds [28-29]. Two functional domains have been identified in trigger factor, (Fig. **1**): residues 1 – 118 constitute the ribosome binding domain [30], while residues 145 – 251 constitute the PPIase domain [31-32]. This latter domain exhibits weak homology to members of the FK506-binding protein (FKBP) family [33]. Al third C-terminal domain,

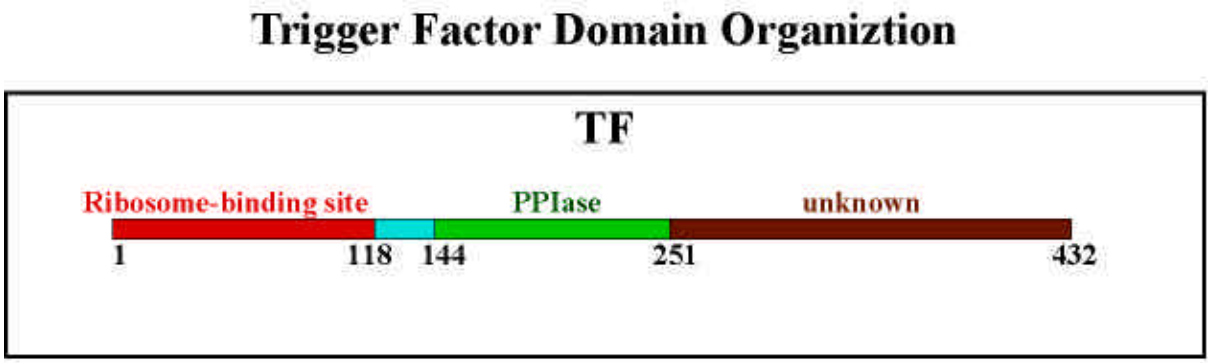

Fig. (1). Domain organization of trigger factor. The domain boundaries of the ribosome-binding site and the peptidyl-prolyl isomerase region of trigger factor are shown.

residues 252 – 432, has also been identified by limited proteolysis [34]. The function of this domain is not known; however, strong interactions have been observed between this domain and the N-terminal domain. These interactions are necessary for the stability of trigger factor. It is important to note that all three domains are required for the high folding activity of trigger factor. This activity is significantly reduced, by a factor of almost 1000, when the N- or C-terminal regions are removed [34]. Thus the PPIase domain in isolation is a poor folding catalyst. The high folding activity of full-length trigger factor depends on the tight binding of TF to substrate proteins and this requires all three domains of the chaperone [28]. It should be noted that the recognition of substrates by trigger factor is found to be independent of the presence of proline residues in these substrates [35]. Hence, trigger factor is not only a prolyl isomerase but also a general folding catalyst. Recently, Huang et al. [36] found that trigger factor competitively binds *in vitro* only to protein substrates with loose tertiary structure. This result is in agreement with the ability of trigger factor to bind to newly-translated nascent chains *in vivo* which are at the early stages of folding.

Trigger factor differs from other chaperones, in that it is not a heat shock protein but rather seems to be induced upon cold shock, and it enhances cell viability at low temperatures [37]. Furthermore, trigger factor does not seem to be able to prevent the aggregation of some typical model chaperone substrates used in *in vitro* studies such as firefly luciferase [38]. In addition, unlike other chaperones, trigger factor is not an ATPase. Its function is not regulated by ATP binding and hydrolysis as other typical chaperone systems. The functional cycle of TF is not yet elucidated.

In summary, trigger factor can be thought of as a ribosome-associated folding factor which accelerates prolyl isomerization and stabilizes nascent chains. It either assists in the folding of these chains or targets them to other chaperone systems.

DNAK/DNAJ/GRPE

Assisting the Folding of Polypeptides in Extended Conformations

After interacting with trigger factor, nascent chains released from the ribosome in *E. coli* can either fold to their native state unassisted or might require further assistance by other chaperone systems. These newly-released polypeptides are probably at an early stage of folding and, hence, expose unfolded polypeptide segments. The major chaperone system in the *E. coli* cytoplasm which is capable of recognizing unfolded polypeptide segments is the Hsp 70 system [39]. This system consists of DnaK (Hsp 70), its cofactor DnaJ (Hsp 40), and a nucleotide exchange factor GrpE. All three proteins are induced by heat shock, however, only DnaK has ATPase activity. The *dnaK* and *dnaJ* genes form an operon with the structure promoter-*dnaK-dnaJ.*

The *dnaK* and *dnaJ* genes were originally identified as essential genes required for the bacteriophage λ growth [40-42]. Subsequent genetic and biochemical studies suggested that DnaK and DnaJ are necessary for the initiation of λ DNA replication [43-44]. Similarly, *grpE* was identified as yet another gene which was required for λ growth [45-46]. It was demonstrated that mutations in the *grpE* gene are responsible for both the inability of λ to replicate at any temperature tested and for the lack of bacteriophage colony formation at high temperatures [47]. These early observations were then followed by extensive biochemical and biophysical studies which established direct physical interaction between DnaK and GrpE [48], and between DnaK and DnaJ [49]. All the experimental data support the proposal that DnaK, DnaJ, and GrpE function together as part of the same chaperone system.

The Hsp 70 chaperone system has many different functions in the cell associated with its involvement in a large variety of protein folding processes. It functions as a general chaperone system that catalyzes protein folding and that promotes the assembly or disassembly of oligomeric protein complexes [50]. Furthermore, *E. coli* strains deleted of *dnaJ* or *dnaKJ* genes are defective in the secretion of several enzymes [51] pointing to a role for this system in protein transport to the *E. coli* periplasm [52]. Genetic evidence, on the other hand, indicates that DnaK is involved in cell division [53] and in the synthesis of RNA and DNA after exposure of cells to heat shock [54-55]. DnaK has also been implicated in directing abnormal proteins to the degradation machinery [56] and in controlling the activity of other regulatory proteins such as RepA, the initiator protein for plasmid replication [57]. Both DnaK and DnaJ have been shown to autoregulate the heat shock response by directly binding to σ^{32} [58-59], the heat shock transcription factor,

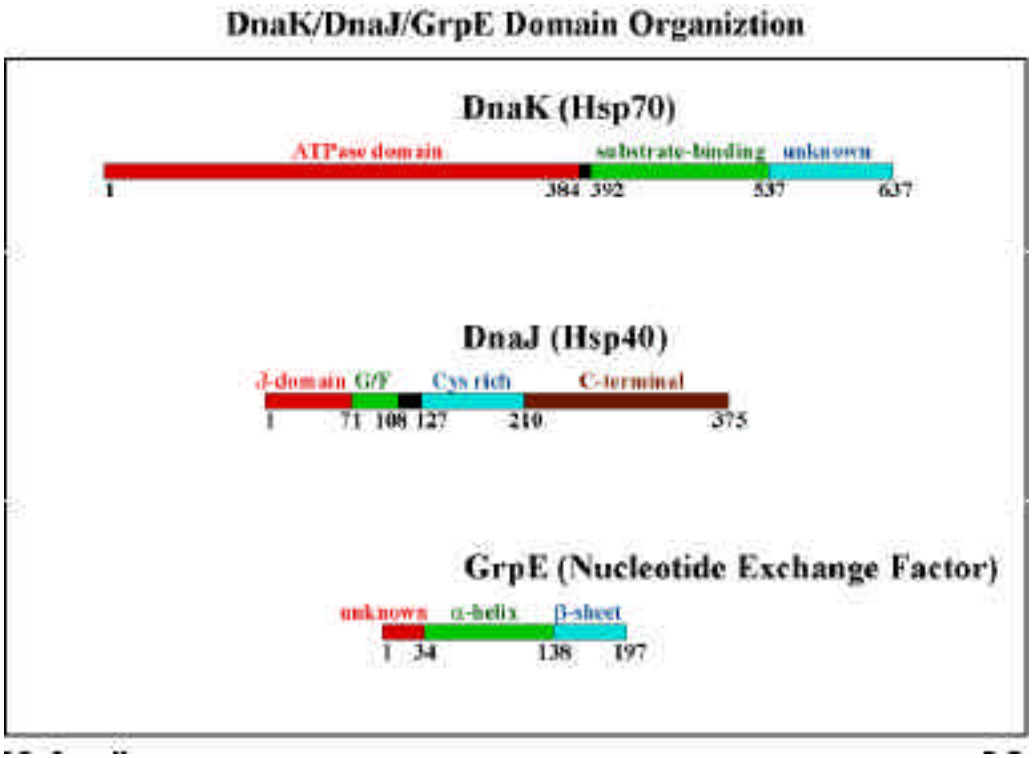

Fig. (2). Domain organization of the Hsp 70 system. The domain boundaries of *E. coli* chaperone DnaK (Hsp 70), its cochaperone DnaJ (Hsp 40), and the nucleotide exchange factor GrpE are shown.

and targeting it for degradation by the inner-membrane-associated protease FtsH [60].

DnaK

The domain arrangement and the structures of DnaK, DnaJ, and GrpE are shown in (Fig. **2**) and (Fig. **3**). DnaK primarily exists as a monomer, although there are some indications that it can also form a dimer at high buffer or protein concentrations [61]. The chaperone chain can be divided into two main domains: an N-terminal ATPase domain (residues 1 – 384) and a C-terminal domain which includes a polypeptide-binding subdomain (residues 392 – 536) and another subdomain of unknown function (residues 537 – 637) [62]. The ATPase domain of DnaK has an actin-like structure [63] consisting of two large lobes with a deep cleft between them. ATP binds at the base of this cleft [64-65]. The binding of DnaK to target polypeptides occurs through a channel defined mainly by loop regions in the C-terminal domain of DnaK [66] and a long α-helix. The preferred peptide-binding motif of DnaK is proposed to consist of an extended linear stretch of 5 hydrophobic residues that are flanked on both sides by regions enriched in basic residues [67].

There are two functional states for DnaK depending on the phosphorylation state of the bound nucleotide [68-72]: a low affintiy ATP-bound state (T) which is characterized by fast-binding and release of substrate polypeptide, and a high affinity ADP-bound state (R) characterized by slow-binding and release of target polypeptide. The conversion from the T state to the R state is mediated by DnaJ and bound substrate, both of which stimulate the ATPase activity of DnaK, while the conversion from the R state to the T state is mediated by GrpE which facilitates the exchange of DnaK-bound ADP for ATP. DnaK binds ADP more tightly than ATP [73]. The chaperone seems to undergo a conformational change when switching between these two states [74], however, this conformational change is not yet understood.

There has been some controversy regarding the *in vivo* role of DnaK. Under normal growth conditions, deletion of the *dnaKJ* gene in a wild-type strain results in lethality [25], while, in other strain backgrounds or under certain conditions, the deletion results only in the rapid accumulation of suppresser mutations [25-75-76]. On the other hand, under heat stress conditions, DnaK seems to be essential for viability under all genetic backgrounds [76]. DnaK has been shown to assist *in vivo* in the *de novo* folding of 5 – 10% of total soluble *E. coli* proteins at 30°C [25-26], preferentially in the size range of 30 – 75 KDa, including ribosome-bound nascent chains. The extent of DnaK binding to nascent chains is modulated by trigger factor since the deletion of the trigger factor gene results in the doubling of the number of nascent chains interacting with DnaK [25] and in increasing the fraction of shorter chains. Hence, trigger factor and DnaK seem to functionally cooperate to chaperone the folding of nascent chains.

The identity of proteins that utilize DnaK for folding under normal growth conditions remain largely unknown. Only four proteins are currently known to directly interact with DnaK at 30°C [77]. Under heat shock, it is estimated that about 15 – 25% of total soluble protein species show increased aggregation in a Δ*dnaK* mutant strain exposed to 42°C for 60 minutes compared to wild-type cells. Hence, these proteins seem to require the function of DnaK to remain soluble under heat stress conditions either directly by

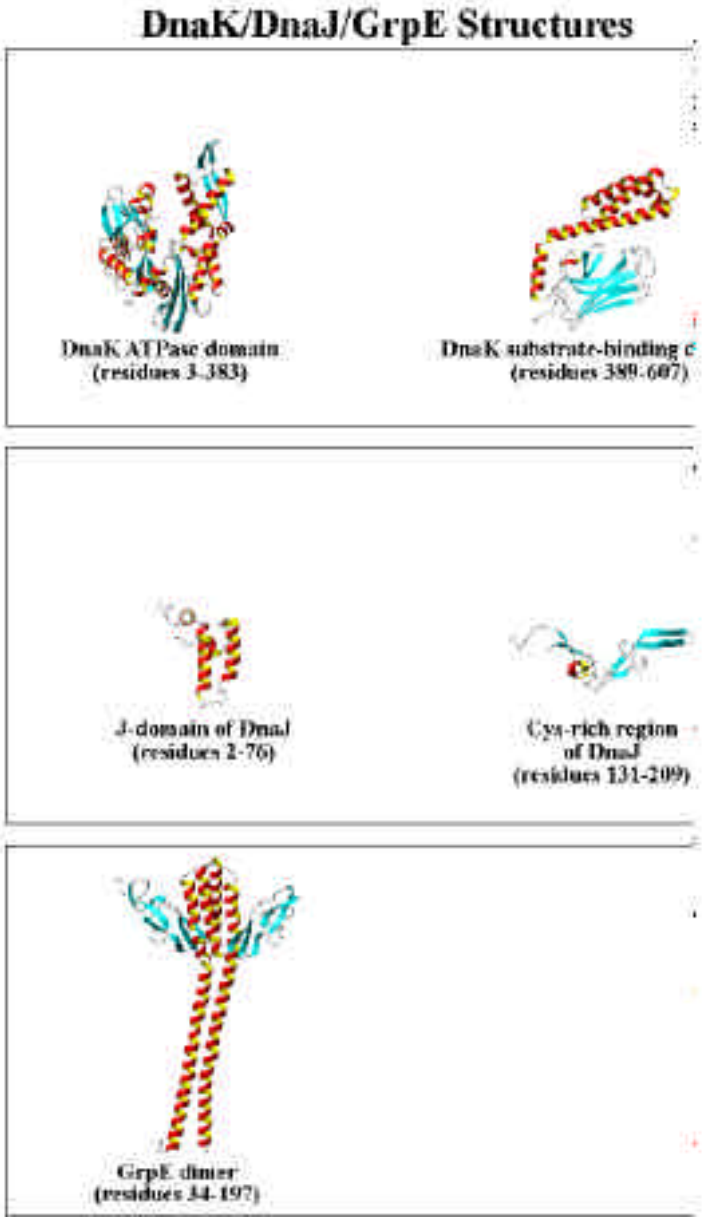

Fig. (3). Structures of the Hsp 70 chaperone machinery. Structures shown are that of the DnaK ATPase domain, DnaK substrate-binding domain, DnaJ J-domain, DnaJ Cys-rich region, and GrpE dimer. Structures are drawn to scale using MolMol [189] and the PDB files: 1DKG [65] for DnaK ATPase domain and GrpE, 1DKX [66] for DnaK substrate-binding domain, 1XBL (model 1) for J-domain of DnaJ [190], and 1EXK for the Cys-rich region of DnaJ [191].

binding to DnaK or indirectly by binding cofactors that require DnaK. Fifty-seven of these proteins have been identified [77]. Not surprisingly, these proteins are preferentially of large molecular weight: 42% of them are above 60 KDa. Large-sized proteins are expected to be vulnerable to thermal unfolding and aggregation since they are typically multidomain proteins which exhibit limited thermodynamic stability.

DnaJ

DnaJ is a modular basic dimeric protein that has at least four distinct domains. It has a J domain which is an evolutionarily highly conserved motif responsible for stimulating the ATPase activity of DnaK [78]. This domain is followed by a glycine and phenylalanine-rich domain which is then followed by a cysteine-rich Zn-binding domain and a C-terminal domain present in most DnaJ homologues. DnaJ functions primarily as a cofactor that enhances the ATPase activity of DnaK, hence, converting the low affinity ATP-DnaK state to the high affinity ADP-DnaK state. The J domain is thought to directly interact with the ATPase domain of DnaK [49] to stimulate ATP hydrolysis by DnaK.

DnaJ itself also possesses molecular chaperone functions since it has been shown to bind to nascent chains in *in vitro* translation systems [79] and to prevent the aggregation of denatured polypeptides [69-80-81]. It has been proposed that the cysteine-rich domain of DnaJ has the ability to recognize

and bind substrate proteins in their denatured state [82]. However, based on structural evidence from the yeast DnaJ homologue (Sis1) [83], it seems that the C-terminal region of DnaJ is the substrate binding site. By screening cellulose-bound peptide libraries, Rüdiger et al. [84] found that the substrate-binding motif of DnaJ consists of a hydrophobic core of about eight residues enriched for aromatic and large aliphatic residues as well as arginine. This motif is similar to that found for DnaK.

GrpE

GrpE is not a chaperone, but rather functions as a nucleotide exchange factor for DnaK [85-86]. GrpE is a very elongated homodimer that binds to DnaK in a ratio of 2:1 [65-87] (Fig. **3**). The dimer interface encompasses two long α helices that lead into a small four helix bundle. Two small β sheet domains emanate from the two C-terminal ends of the four helix bundle. GrpE binds to the ATPase domain of DnaK mainly through the β sheet domains and along separate regions on the long helices. An interaction between GrpE to the substrate-binding domain of DnaK has also been proposed [65-87]. The binding of GrpE to DnaK results in the opening of the nucleotide binding cleft of the ATPase domain of DnaK causing the dissociation of ADP from DnaK. Since ADP-DnaK-substrate complex is very stable, the binding of GrpE to this complex results in the dissociation of ADP from DnaK and the transition of DnaK to the low affinity ATP-DnaK state causing the dissociation of the bound substrate from the chaperone [88-89]. So GrpE serves a dual function for DnaK: as a nucleotide exchange factor and as a substrate release factor.

The Hsp 70 System Functional Cycle

The ATP-dependent functional cycle of the Hsp 70 system can be described as follows (Fig. **4**). When not in complex with DnaJ and substrate protein, a large fraction of DnaK is in the low affinity ATP state (T) [73]. A target polypeptide, whether still attached to the ribosome or just released from the ribosome, is initially bound by DnaJ which recognizes hydrophobic stretches of amino acid residues in the polypeptide primary structure. DnaJ-bound polypeptide is then transferred to the low affinity ATP-DnaK which will also bind to the hydrophobic stretches on the protein chain. Both DnaJ and substrate protein stimulate the ATPase activity of DnaK, this switches the chaperone to the high affinity ADP-DnaK state (R) and results in the formation of a stable ADP-DnaK-substrate complex. DnaK remains associated with the substrate protein until GrpE binding to DnaK results in the dissociation of ADP. This destabilizes the interaction between DnaK and substrate

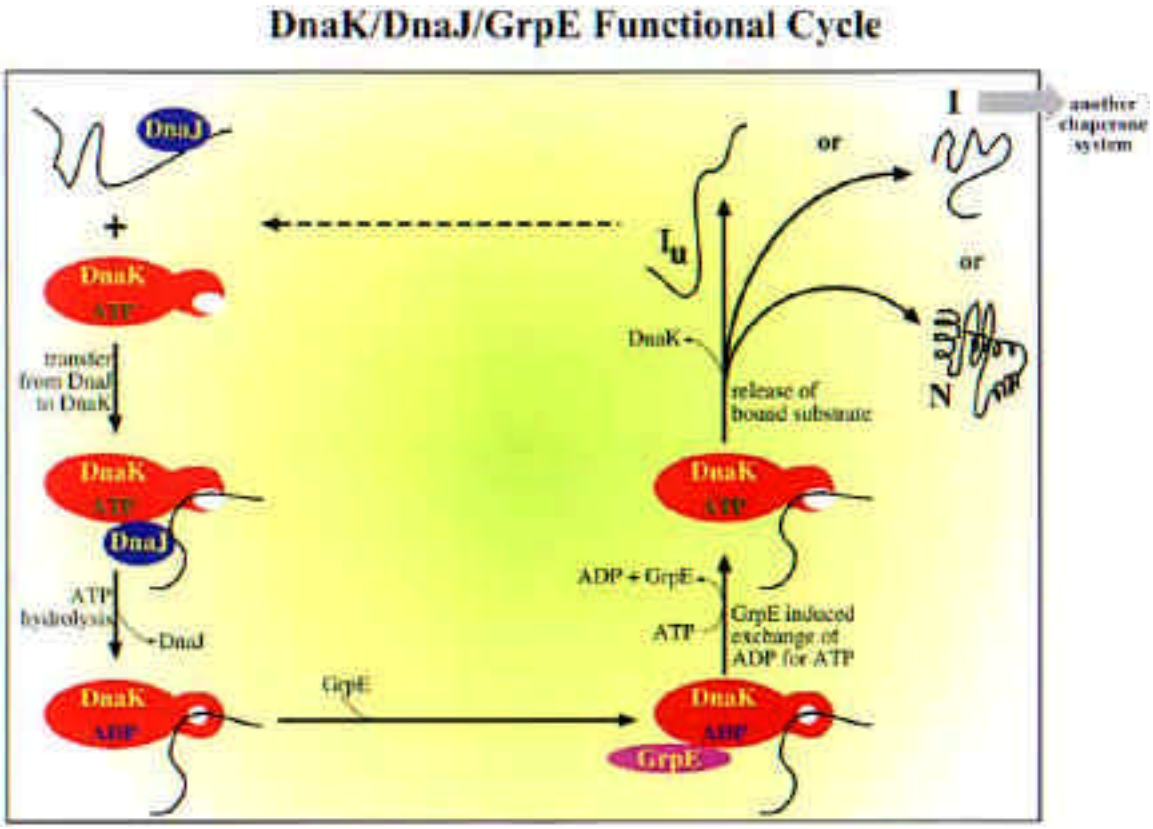

Fig. (4). The Hsp 70 system functional cycle. The different features of the cycle are discussed in the text. A target polypeptide is initially bound by DnaJ. The polypeptide is then transferred to the low affinity ATP-DnaK. Both DnaJ and the substrate protein stimulate the ATPase activity of DnaK, resulting in the formation of a stable ADP-DnaK-substrate complex. GrpE binding to DnaK results in the dissociation of ADP. This destabilizes the interaction between DnaK and the substrate protein causing the release of substrate from the chaperone. The released polypeptide chain can either attempt to fold to its native state or can be rebound by the Hsp 70 system for another cycle of chaperone assisted folding or can be bound by another chaperone system.

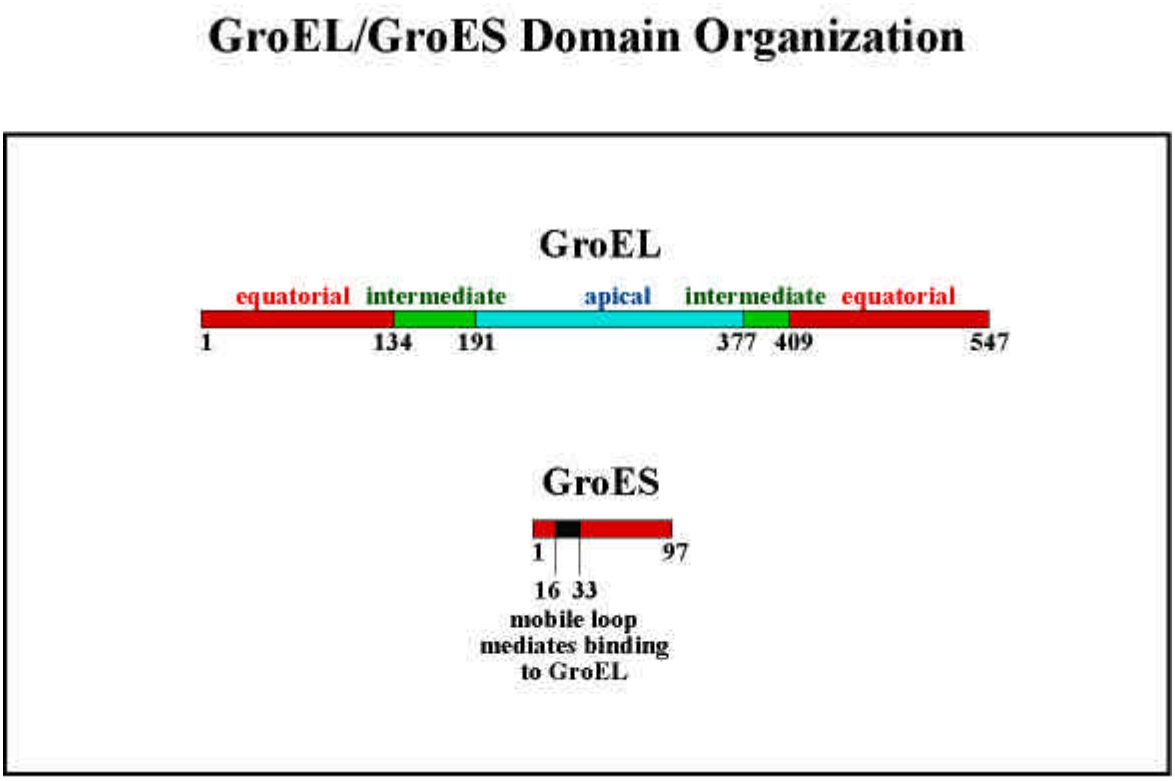

Fig. (5). Domain organization of the Hsp 60 system. The domain boundaries of *E. coli* chaperonin GroEL (Hsp 60) and its cochaperone GroES (Hsp 10) are shown.

protein causing the release of substrate from the chaperone. DnaK then converts to the low affinity ATP-DnaK state. The released polypeptide chain can either attempt to fold to its native state or can be bound by another chaperone system, or can be rebound by the Hsp 70 system for another cycle of chaperone assisted folding. Most proteins seem to require multiple rounds of binding and release by molecular chaperones in order to reach their native states.

The general features of the functional cycle for the *E. coli* Hsp 70 system seem to be conserved in all other Hsp 70 systems in different organisms. The differences might be in the specificity of the different DnaK/DnaJ/GrpE homologues and the presence of other cofactors which might modulate this cycle.

GROEL/GROES

Assisting the Folding of Polypeptides in Compact Conformations

A folding polypeptide chain in a compact conformation can either proceed to its native state unassisted or can be further assisted in its folding by another chaperone system, GroEL/GroES, which recognizes compact intermediates exposing hydrophobic surfaces.

The chaperonin GroEL with its cofactor GroES are the typical representatives of the Hsp 60 and Hsp 10 families of molecular chaperones, respectively. GroEL/GroES is the only essential chaperone system in *E. coli* cytoplasm under all growth conditions [90]. *groES* and *groEL* genes form an operon with the order promoter-*groES-groEL*. GroEL is a homo-oligomer of fourteen, 57 KDa, subunits arranged into two heptameric rings forming a cylindrical structure with two large cavities (Fig. **5**) and (Fig. **6**). GroES is a heptamer of 10 KDa subunits, and it forms a cap over the cavities in the GroEL cylinder. The GroEL subunit is divided into apical (residues 191 – 376), intermediate (residues 134 – 190 & 377 – 408), and equatorial (residues 1 – 133 & 409 – 547) domains. Protein binding takes place at hydrophobic residues located at the outermost apical domain [91]. These residues form a hydrophobic patch at the rim of each of the GroEL rings. The intermediate domain forms a short flexible linker between the apical and equatorial domains, while the equatorial domain forms the contact region between the two GroEL rings. ATP binding and hydrolysis takes place at the equatorial domains. The apical and intermediate domains of GroEL undergo large conformational changes as a result of nucleotide and GroES binding (Fig. **6**).

In Vitro Aspects

The mechanism of GroEL function is well-established *in vitro* [92-95] (Fig. **7**). The substrate-acceptor state of GroEL is thought to be an ADP bullet in which GroES and seven ADPs are bound to the same ring of GroEL. This ring might also contain a previously encapsulated polypeptide. Another substrate protein then binds to the apical domains of the opposite free ring of GroEL in a non-native compact conformation mainly through hydrophobic interactions to hydrophobic residues in helices H (residues 234 - 243) and I (residues 256 - 268) and a loop region (residues 196 - 211)

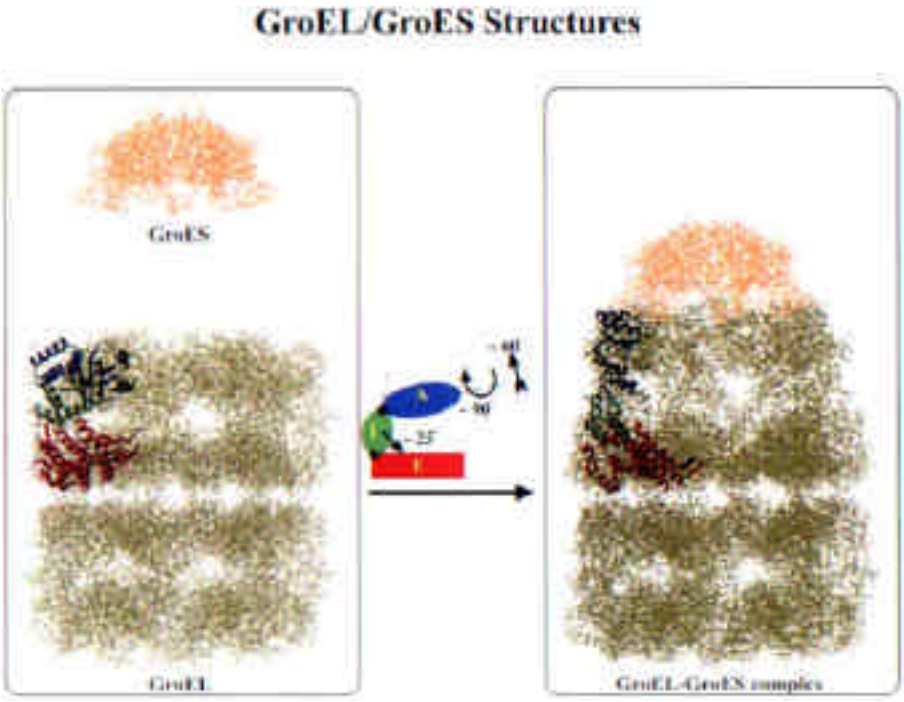

Fig. (6). Structures of the Hsp 60 chaperone machinery. GroEL, GroES and GroEL-GroES complex are displayed using the stick representation. The conformation of a single GroEL subunit in GroEL or in GroEL-GroES complex is shown using the ribbons representation with color coded as follows: apical domain in blue, intermediate domain in green, and equatorial domain in red. The movement of the GroEL subunit as a result of ATP and GroES binding is indicated. Structures were drawn using Swiss-PdbViewer [192] and the PDB files 1DER [193] and 1AON [96].

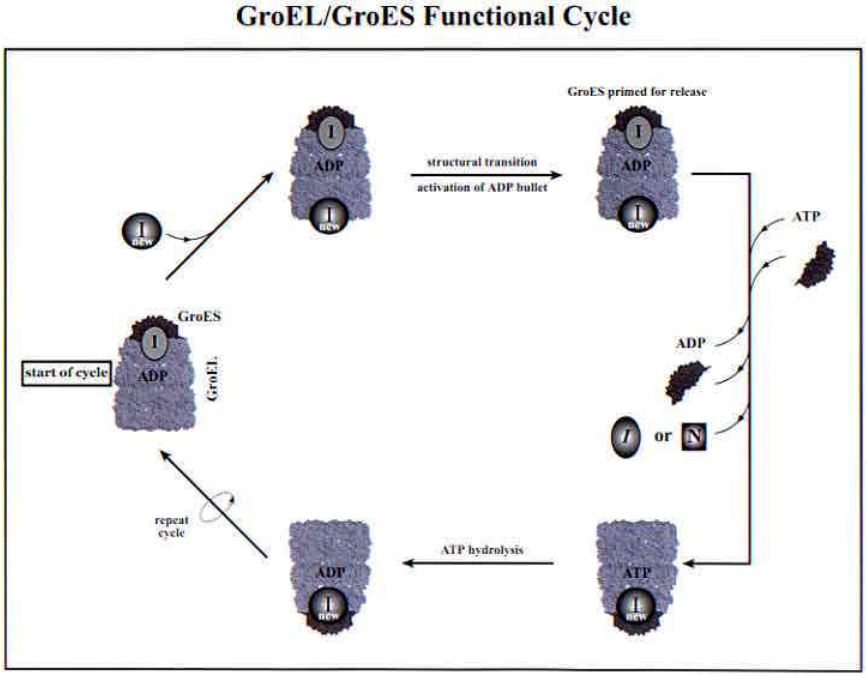

Fig. (7). The Hsp 60 chaperone cycle. The details of the cycle are described in the text. The substrate-acceptor state of GroEL is thought to be an ADP bullet in which GroES and seven ADPs are bound to the same ring of GroEL. A substrate protein binds to the apical domains of the free ring of GroEL in a non-native compact conformation. ATP and then GroES bind to the newly occupied ring of GroEL. This results in the displacement of the bound protein into a chamber defined by the GroEL ring and the GroES cap. This also results in the release, from the opposite ring, of ADP and GroES as well as any previously encapsulated polypeptide. After about 10 – 20 seconds of GroES binding, ATP on the polypeptide-containing ring is hydrolyzed to ADP. This results in the formation of a new ADP bullet which will act as the acceptor state for another substrate molecule. The cycle then repeats as described above.

[91]. In this form, GroEL then undergoes a structural transition into an activated ADP bullet which is primed for the release of GroES. The nature of this structural transition is not yet clear. Subsequently, ATP and then GroES bind to the newly occupied ring of GroEL resulting in concerted upwards and outwards movement of the apical domains of GroEL relative to the intermediate hinge regions generating a large enclosed chamber (see Fig. **6**). This results in the displacement of the bound protein into this new chamber defined by the GroEL cylinder and the GroES cap. This also results in the release, from the opposite ring, of ADP and GroES as well as any previously encapsulated polypeptide. GroES binding to GroEL is mediated through a mobile loop in GroES which directly interacts with helices H and I of GroEL [96]. The folding process of the new substrate then takes place in the enclosed cage in which aggregation and other possible unproductive interactions are prevented. After about 10 – 20 seconds of GroES binding, ATP on the polypeptide-containing ring is hydrolyzed to ADP. This results in the formation of a new ADP bullet which will act as the acceptor state for another substrate molecule. The cycle then repeats as described above. Most proteins seem to require multiple rounds of binding and release to reach their native state.

There is some controversy regarding the role that GroEL plays in the actual folding process of substrate proteins. GroEL is usually observed to retard the folding of proteins which can fold rapidly *in vitro* [97-99]. It has also been observed to accelerate the refolding rate of those proteins which fold slowly and are prone to aggregation [100-102]. Furthermore, it has been proposed that GroEL can actively unfold substrate proteins during its functional cycle [103-104]. In any case, there seems to be a general consensus that, even though GroEL might affect the folding rate of proteins, it does not change the folding mechanism of these proteins [105]. It only provides a folding-cage without contributing any steric information to the folding process itself.

In Vivo Aspects

In order to investigate how newly-translated proteins utilize GroEL for folding, the kinetics of passage of these proteins through GroEL (flux kinetics) was followed by carrying out pulse-chase experiments on *E. coli* cells in mid-log phase under normal and heat shock conditions [106-107]. After the chase, GroEL-substrate complexes were isolated and separated on one dimensional or two-dimensional gels. Based on these experiments, the following major conclusions were reached. (1) Three classes of proteins are present in the cell cytoplasm. (a) Proteins with a chaperonin independent folding pathway. (b) Proteins with an intermediate chaperonin dependence for which normally only a small fraction transits GroEL. (c) Highly chaperonin-dependent proteins which require sequestration of aggregation-sensitive intermediates within the GroEL cavity for successful folding. (2) About 300 proteins, representing 10 – 15% of total cytoplasmic *E. coli* proteins, utilize GroEL for *de novo* folding under normal growth conditions and about twice as much under heat stress conditions. (3) GroEL preferentially interacts with proteins of molecular weight between 20 – 60 KDa, as compared to cytoplasmic *E. coli* proteins. About two-thirds of these proteins flux through GroEL with time constants between 20 seconds and 2 minutes, reflecting the requirement of one to several rounds of GroEL binding and release to reach their native state. For the other substrates, a fraction of the population of a particular protein remain associated with GroEL after the chase. This later set of proteins represents pre-existing proteins that require repeated cycles of GroEL binding and release for conformational maintenance during their lifetime in the cell. This set of proteins were found to be relatively unstable *in vivo*, since, under heat shock, the same set interacted more extensively with the chaperonin. Hence, GroEL is important for folding newly-translated proteins as well as for conformational maintenance of pre-existing proteins.

Substrate Recognition by GroEL

Although purified GroEL is able to bind *in vitro* to ~50% of soluble *E. coli* proteins in their unfolded or partially folded states [108], the finding that only ~300 proteins require the chaperonin for folding *in vivo* indicates that GroEL, in the cell, preferentially recognizes features present only in this small fraction of proteins. A proteomics/bioinfomatics approach was employed in order to identify a large fraction of GroEL substrates. 52 proteins where identified using this procedure [107]. Substrates included essential components of the transcription/translation machinery as well as enzymes involved in several metabolic pathways.

Sequence analysis failed to reveal statistically significant consensus sequences among the 52 identified substrates in agreement with *in vitro* studies which also did not find a consensus sequence among model peptides that bound to GroEL [17]. However, structural classification of substrate domains using domain classification databases such as SCOP [109] or CATH [110] demonstrated that GroEL substrates preferentially contained two or more αβ domains compared to soluble *E. coli* proteins. The most common domain architectures in GroEL substrates were those of the three-layer αβα and the two-layer αβ sandwiches. These domains typically have α helices and buried β sheets with extensive hydrophobic surfaces. These surfaces are ideally suited for recognition by the hydrophobic residues in the apical binding domains of GroEL.

The apical domains of GroEL can assist in the folding of such multidomain αβ proteins in several ways. Residues implicated in polypeptide binding in the apical domains of GroEL can provide a scaffold on which the hydrophobic β-sheet(s) or amphipathic helices of the substrate protein can assemble; subsequent folding of the protein can then proceed in the enclosed GroEL-GroES cavity. Alternatively, if a wrong or improper packing occurred between helices and β-sheet(s) in a protein, the apical domains of GroEL could interfere by displacing the helices and β-sheet(s) and then binding to the exposed hydrophobic surfaces. Upon GroES binding to GroEL, the apical domains would then be released from these hydrophobic faces, and the protein would attempt to refold to achieve the right packing interactions. In this regard, it has been demonstrated that the binding of unfolded rhodanese, malate dehydrogenase, and rubisco to the chaperonin requires recognition by multiple GroEL apical

domains [111]. All three proteins are stringent substrates of GroEL, requiring GroES and ATP for efficient folding, and all three consist of two αβ domains.

Further insight into the mode of binding of GroEL to its substrates has been obtained from structural studies. Recent structural data have demonstrated that only those peptides with a high tendency to present a hydrophobic surface exhibit high affinity for GroEL binding irrespective of whether the peptide adopts a helical or a strand conformation [112]. In the X-ray structure of the apical domain of GroEL (residues 191-376), Buckle et al. [113] observed that seven residues in the N-terminal tag of one molecule bind through hydrophobic interactions to helices H and I of a second molecule in a mainly extended conformation. More recently, Chen and Sigler [114] solved the crystal structure of the apical domain of GroEL in complex with a model peptide. They found that the peptide bound to helices H and I in a β hairpin conformation. Although no helical substrates have yet been seen bound to GroEL at atomic resolution, however, transferred nuclear Overhauser enhancements experiments have demonstrated that some peptides adopt a helical conformation when bound to GroEL [115-116]. These different structural studies reveal the ability of the apical domain of GroEL of binding to a wide range of non-native substrates provided a hydrophobic surface is available for binding.

CLP ATPASES

Assisting in Unfolding Proteins and in Disaggregating Protein Aggregates

The Clp ATPases are a unique group of ATP-dependent chaperones associated with the assembly and disassembly of protein complexes. They are members of the AAA family of proteins which are ATPases Associated with a variety of cellular Activities [117]. The key feature of this family is a highly conserved AAA module of about 230 amino acids present in one or two copies in each protein. Each module has a conserved Walker A motif, involved in binding the phosphate of ATP, and Walker B motif, involved in metal binding and ATP catalysis [118]. AAA modules are present in all kingdoms of life and they are involved in many cellular functions [119]. They act as regulatory subunits of proteases [120], they prime the assembly of various membrane-targeting protein complexes during membrane fusion [121], they are involved in DNA replication and recombination [122], and microtubule regulation [123]. The unifying structural feature of these AAA proteins is the arrangement of the subunits into ring-shaped hexameric or heptameric complexes [124-127]. By using whole genome analysis, iterative database searches, and multiple sequence alignments, it has been shown that the AAA family is actually a subset of a much large AAA^+ family [128].

The main Clp ATPases in *E. coli* cytoplasm are ClpA (758 aa), ClpB (857 aa), ClpX (423 aa), ClpY (443 aa) - also known as HslU (Fig. **8**). All of these chaperones are considered to be members of the Hsp 100 family, although ClpA is not induced upon heat shock. Both ClpA and ClpB have two AAA modules (class I), while ClpX and ClpY have only one (class II) which is homologous to the second AAA module in ClpA and ClpB [118]. Although all four proteins can function alone as molecular chaperones (see below), ClpA, ClpX, and ClpY have also been found to function as ATP-dependent regulatory components for proteases ClpP and ClpQ (also called HslV) [129]. In this regard, it is interesting to note that, *clpX* and *clpP* genes form an operon with the order promoter-*clpP*-*clpX* [130-131]; similarly, *clpY* and *clpQ* genes form an operon [132] with

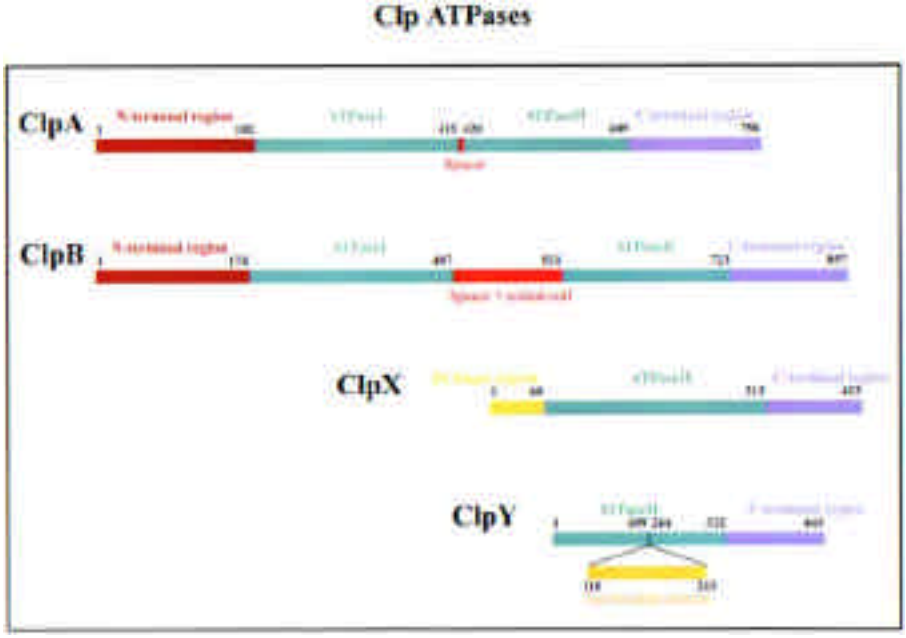

Fig. (8). Domain organization of the Clp ATPases. The domain boundaries of *E. coli* ClpA, ClpB, ClpX, and ClpY are shown. ClpA and ClpB contain two AAA domains, while ClpX and ClpY have only one such domain.

the order promoter-*clpQ-clpY*. The ClpP and ClpQ proteases are not members of the AAA family; the naming in this case is a bit misleading. ClpP is a serine protease [133] and is a homo-oligomer of 14 subunits which associate into two rings with seven fold symmetry [134], while ClpQ is a threonine-dependent protease [135] and is a homo-oligomer of 12 subunits which associate into two rings with six fold symmetry [126]. Neither ClpP nor ClpQ have ATPase activity.

Biochemical and structural studies, have shown that hexameric ClpX or hexameric ClpA can bind to tetradecameric ClpP protease [134-136-137] to form $ClpX_6$-$ClpP_{14}$-$ClpX_6$ and $ClpA_6$-$ClpP_{14}$-$ClpA_6$ complexes, respectively (Fig. **9**). This results in a complex with a symmetry mismatch that might have functional consequences (see below). The structure of ClpP alone has also been solved by X-ray crystallography [134], while the structures of ClpAP and ClpXP complexes have been observed by electron microscopy [136-138] (Fig. **9**). The micrographs show that ClpP is composed of two rings with seven subunits superimposed in bipolar fashion with seven fold symmetry. ClpP is flanked on one or both sides by ClpA or ClpX. ClpX appears as a hexameric ring with a fuzzy mass on one side which might correspond to the N-terminal Zn finger region. ClpA, on the other hand, appears as a bilobed hexameric ring. Each lobe might contain one of the two AAA domains of ClpA. There is also a fuzzy mass on one side of the ClpA ring which might be attributed to the N-terminal domain of the protein. On the other hand, hexameric ClpY binds to hexameric ClpQ protease [126-139] to form $ClpY_6$-$ClpQ_6$-$ClpY_6$. ClpB has not yet been found to interact with any protease components.

ClpA and ClpX

Both ClpA and ClpX were initially discovered as specificity factors which stimulate the peptidase activity of the protease ClpP [130-131-140-141]. ClpP alone can digest small peptides in the absence of ClpA and ClpX, however, it cannot digest large peptides or proteins. It has been shown that the complex ClpAP or ClpXP is required for the processive degradation of larger polypeptides [122]. This degradation process is dependent on ATP binding and hydrolysis. Since ClpP does not bind ATP, it is the energy derived from the ATPase of ClpA and ClpX which seems to be required to unfold substrate proteins and to target them for degradation by ClpP. Because of the symmetry mismatch between ClpA or ClpX (both are hexamers) and ClpP (which is made of two heptamers), it was proposed initially that there is a rotation of ClpA or ClpX relative to ClpP upon ATP hydrolysis. This cork-screw effect is postulated to facilitate the movement of protein substrates into the cavity

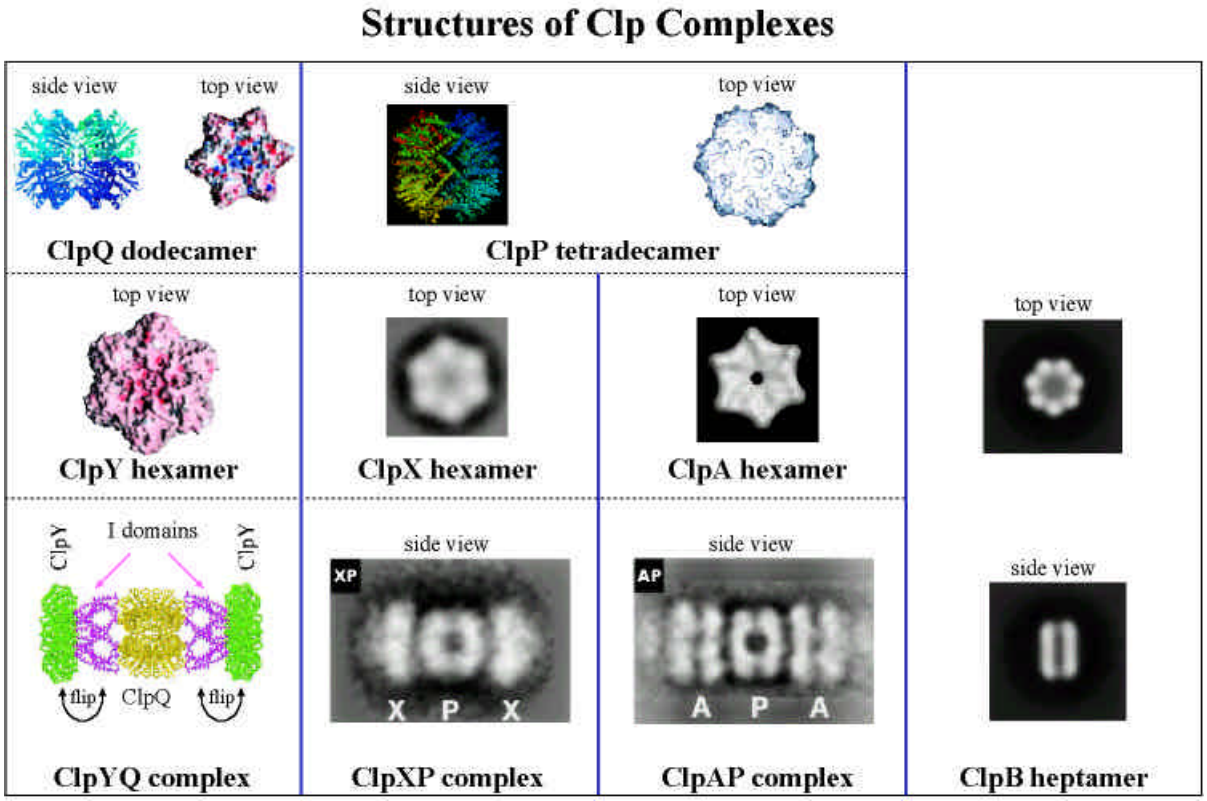

Fig. (9). Structures of the *E. coli* Clp ATPases. The crystal structures of ClpQ, ClpY, and ClpYQ [126-139-194] are shown. The I domains in ClpY are the protrusions that come down from ClpY and interact with ClpQ. However, there are indications that the I domains might actually be pointing away from ClpQ. Also shown is the crystal structure of ClpP [134]. In addition, the electron micrographs of ClpX [136], ClpXP [136], ClpA [138], ClpAP [136], and ClpB [181] are displayed. ClpB in these micrographs is observed to be a heptamer, however, there are other reports indicating that it might be a hexamer or a tetramer (see text). All figures are obtained from the cited references.

of ClpP [138]. However, more recent data do not show such an effect, but rather indicate that ATP hydrolysis is required by ClpA and ClpX to actively unfold substrate proteins and to translocate them to ClpP [142-145].

Both ClpA and ClpX have chaperone activities. ClpA can remodel inactive dimers of the plasmid P1 initiator protein, RepA, into active monomers that can specifically bind to DNA [146]. This reaction requires ATP binding and hydrolysis by ClpA. One cycle of RepA binding to ClpA followed by ATP-dependent release is sufficient to convert inactive RepA to its active form [147]. ClpA can also prevent the irreversible heat inactivation of proteins, however, it is unable to actively induce their reactivation [146]. ClpX, on the other hand, has been shown to be involved in the replication of bacteriophage Mu DNA. It seems to catalyze the disassembly of the hyperstable complexes of MuA and transposase tetramers bound to DNA in the presence of ATP [148]. ClpX alters the conformation of the DNA-bound MuA protein and converts the MuA-DNA complex to a less stable form [149]. In addition, ClpX can protect the bacteriophage λO replication protein from heat-induced aggregation in an ATP dependent manner [150]. It can also dissolve preformed λO aggregates [150].

Both ClpAP and ClpXP are involved in regulating the levels of several *E. coli* proteins. ClpAP has been shown to be required for the degradation of the N-end rule substrates in *E. coli* [151]. The N-end rule relates the *in vivo* half-life of a protein to the identity of its amino-terminal residue [152]. ClpXP has been shown to be involved in the degradation of stationary phase sigma factor [153-154] σ^s, which regulates the expression of stationary phase and stress response genes. σ^s is rapidly degraded during exponential growth phase and much more slowly during stationary phase. Another *E. coli* protein whose levels are also regulated by ClpXP is UmuD′ [155-156]. This protein is a mutagenically active form of UmuD and is part of the DNA pol V complex, UmuD′$_2$C, which catalyzes the synthesis of nascent DNA opposite normally replication-blocking lesions [157-158]. These lesions occur when *E. coli* cells are exposed to extensive DNA damage. Unlike normal replication, synthesis of DNA by pol V is highly error prone, resulting in a large increase in mutation rates. Cells use error prone DNA synthesis only as a last resort for survival. Hence, the activity of pol V is maintained to a minimum and the levels of its different subunits are highly regulated by ClpXP and other proteases.

Both ClpAP and ClpXP also play important roles in the degradation of SsrA-tagged proteins. When ribosomes are stalled in *E. coli*, a unique SsrA RNA (also called tm RNA or 10Sa RNA) acts both as tRNA and mRNA to clear these ribosomes of attached nascent chains. The chains are tagged with a C-terminal eleven-residue peptide that targets them for degradation [159]. The tag, which is termed an SsrA tag, carries a hydrophobic signal (AANDENYALAA) that is specifically recognized by ClpAP and ClpXP [160] as well as by other periplasmic proteases [159]. Recently, it has been demonstrated that there is a directional transfer of SsrA-tagged substrates from ClpA to ClpP with the COOH terminus of the substrate protein entering ClpP first [161]. Evidence is currently emerging that the tagging system involves a large ribonucleoprotein complex [162] which includes the SsrA RNA, a unique RNA-binding protein termed SmpB [163], and a host of many other cofactors. Furthermore, a ribosome-associated protein, termed SspB, has recently been found to bind specifically to SsrA-tagged proteins and to enhance their recognition by ClpXP. Hence, SspB functions as a specificity-enhancing factor for ClpXP. The specificity by which ClpA and ClpX recognize the SsrA tag has recently been demonstrated for the model green fluorescent protein (GFP). GFP is a very stable protein *in vitro*. However, if it is C-terminally tagged with SsrA peptide, ClpA and ClpX can bind and actively unfold this protein in an ATP dependent fashion. In the absence of this tag, the Clps have no effect on GFP conformation [142-145].

ClpY (HslU)

The *clpQY* operon was first identified as part of a study aimed at finding new heat shock genes in *E. coli* [164]. ClpQ protease provides a surprising link to the eukaryotic proteasome. It shares around 20% sequence similarity [165] and a conserved fold with the 20S eukaryotic proteasome β-subunits [166]. ClpY on the other hand, is a close homologue of ClpX and functions to enhance the peptidase activity of ClpQ in an ATP-dependent manner [132-167]. ClpYQ has been found to be involved in the general proteolysis of misfolded proteins, and it also seems to be more specifically involved in regulating the levels of the heat shock transcription factors σ^{32} and σ^E [168-169].

Another protein whose levels are regulated by ClpYQ is the cell division inhibitor protein SulA [170-171]. The synthesis of SulA is induced by DNA damage as part of the SOS response in *E. coli*. SulA functions to inhibit cell division by binding the essential cell division protein FtsZ. Wild-type SulA is unstable and has a tendency to aggregate. In this regard, it has been shown that ClpY *in vitro* functions as a typical molecular chaperone to prevent the aggregation of SulA in a concentration-dependent manner [172]. Furthermore, the expression of ClpY in a Δ*clpQY* strain stabilized SulA *in vivo* and enhanced its ability to block cell division [172]. Hence, it seems that ClpY has two opposing functions: one as a chaperone promoting the stability and preventing the aggregation of SulA and another one as a regulatory component of the ClpQ protease supporting the degradation of SulA.

The crystal structure of *E. coli* ClpYQ has recently been solved [126]. This structure has caused some controversy in the field. The ClpQ protease appears to be made of two rings of six subunits each having six fold symmetry (see Fig. **9**). ClpY forms a hexameric ring packed on both sides of ClpQ. Each ClpY subunit consists of three domains: an N-terminal domain containing the AAA module, a C-terminal domain, and a flexible intermediate (I) domain which emerges from the N-terminal domain (Fig. **8**). In the crystal structure of *E. coli* ClpYQ, the I domain interacts with the protease, while in the crystal structure of the homologous protein complex from *Haemophilus influenzae* the I domain points away from the protease [139]. Furthermore, pictures taken of *E. coli* ClpYQ by cryo-electron microscopy, show that the I domains are exposed on the distal surfaces of ClpY pointing away from the protease [173]. The images are similar to those taken of ClpXP. Hence, the I domain in ClpY might

play a role similar to the Zn finger region of ClpX. Consequently, the I domain might be the substrate binding site in ClpY rather than the protease binding region.

ClpB

ClpB was initially identified as a heat shock protein with close homology to ClpA [174-175]. ClpB has two AAA domains [refer to Fig (**9**)] and its ATPase activity is stimulated by the presence of substrate proteins such as casein [176]. However, unlike ClpA, ClpB does not associate with any protease components. *E. coli* cells deleted of *clpB* show a slower growth rate at 44°C and a higher rate of death above 50°C [175-177] indicating that ClpB is essential for cell survival at high temperatures.

The *clpB* gene has been shown to contain dual initiation sites for translation which results in the synthesis of a ~95 KDa and a ~80 KDa ClpB polypeptide [178]. The 95 KDa polypeptide corresponds to the wild-type protein, while the 80 KDa polypeptide is missing the N-terminal domain. Both polypeptides have similar inherent ATPase activities and adopt similar oligomeric states when run on a gel filtration column. However, unlike the larger polypeptide, the ATPase activity of the 80 KDa ClpB does not seem to be stimulated by substrates. The observation led to the proposal that the N-terminal region of ClpB (residues 1 – 149) contains the substrate binding site [178]. This was further supported by truncation studies [179].

The C-terminal region of ClpB, on the other hand, seems to be mainly involved in the oligomerization of the protein. Initially, it was proposed that ClpB is a tetramer at low salt concentrations [176]. It was subsequently shown that under conditions with 0.2 M KCl, ClpB behaves as a monomer in the absence of ATP and as a hexamer in its presence [180]. Recently, it was demonstrated by electron microscopy that ClpB assembles into heptamers either in the absence of salt and ATP or in the presence of high salt concentrations (> 0.2 M NaCl) and ATP [181]. So, the physiological oligomeric state of ClpB is still a matter of controversy.

The chaperone activity of ClpB has been demonstrated by its ability to reactivate urea denatured firefly luciferase in conjunction with the Hsp70 system (DnaK, DnaJ, GrpE) [182]. It should be emphasized that ClpB itself does not seem to assist in protein folding, but rather it functions to disaggregate preformed protein aggregates and then to transfer these proteins to the Hsp 70 chaperone system [77-183] which subsequently promotes their refolding. The mechanism by which ClpB accomplishes this task is not yet understood. Indeed, the mechanism by which any of the Clps perform their function is not yet known and remains to be addressed.

CHAPERONE NETWORKS

Evidence for the presence of chaperone networks comes from the ability of the cell to compensate for the depletion of one chaperone system by overexpression of other chaperones. This has been demonstrated especially for chaperones DnaK [76] and GroEL [184]. The coordination between different chaperone systems might be due to a direct effect of the chaperones on the heat shock transcription factor σ^{32} resulting in the overexpression of all heat-shock-induced chaperones or as a general response to an increased accumulation of misfolded proteins in the cell.

In addition, there is also evidence that these chaperone networks are further organized in a manner by which there is a preferred sequence of interaction of chaperones with newly-synthesized proteins. In prokaryotes, since trigger factor modulates the binding of DnaK to nascent chains and since trigger factor binds to shorter nascent chains than DnaK [25], then trigger factor seems to act on newly-emerging nascent chains prior to DnaK. Furthermore, since DnaK recognizes polypeptide segments in extended conformation while GroEL recognizes compact intermediates, and, since DnaK has the ability to bind to ribosome-associated nascent chains while no such binding has been observed for GroEL, it is reasonable to assume that DnaK acts in the cell at an earlier stage in the folding of target proteins than does GroEL. Hence, the sequence of interactions of newly-synthesized proteins with chaperones is proposed to be trigger factor, then the Hsp 70 system, and then the Hsp 60 system. Such a sequence of interaction implies that there is a direct transfer of some substrates from one chaperone system to the other. A direct transfer has indeed been demonstrated *in vitro* [80-185] and has been suggested to occur *in vivo* [25-186]. It should be noted that the possibility of back transfer from one chaperone system to another is not strictly excluded. Such a back transfer from GroEL to DnaK has been demonstrated to occur *in vitro* for a large protein that does not fit in the GroEL-GroES cavity [187].

In addition, it has been recently shown that the disaggregation and refolding of preformed protein aggregates involves a direct transfer from ClpB to the Hsp 70 chaperone [77-183] – at least *in vitro*. In these studies it was shown that ClpB initially binds directly to preformed protein aggregates. Subsequently, ClpB undergoes an ATP-dependent conformational change that increases the exposure of hydrophobic patches in the aggregates. These patches are then recognized by the Hsp 70 system which binds to the aggregates and mediates their refolding. This bichaperone mechanism of disaggregation and refolding can catalytically reactivate a wide spectrum of natural substrates, albeit with various efficiencies which seems to depend on the nature and size of the aggregates [188].

CONCLUDING REMARKS

The general principles governing chaperone-assisted protein folding seem to be conserved among the different species. A lot is now known about the different chaperone systems in prokaryotes and of their homologues in eukaryotes. However, there are many key questions that remain to be addressed. The substrates of the different chaperone systems are not known and need to be identified. This will shed a lot of insight into the functional organization of the chaperone machinery in the cell. The mechanistic analysis of the Clp ATPases is still lacking, partly due to the difficulty in working with purified Clps. Finally, the link between folding and degradation needs to

be addressed since the question of how the cell knows that a given misfolded protein needs to be disaggregated and folded rather than degraded remains so far unanswered.

LIST OF ABBREVIATIONS

Hsp	=	Heat shock protein
TF	=	Trigger factor
Nascent chain	=	Newly-synthesized polypeptide chain still attached to the ribosome
PPIase	=	Peptidyl-prolyl cis-trans isomerase
Chaperonin	=	Refers specifically to the Hsp 60 chaperones such as GroEL
GFP	=	Green fluorescent protein

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