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The Hsp70 and Hsp60 Chaperone Machines

Review

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An essential cellular machinery that has been identified and studied only relatively recently is a collective of specialized proteins, molecular chaperones, that bind nonnative states of other proteins and assist them to reach a functional conformation, in most cases through the expenditure of ATP. Originally identified by their increased abundance following heat shock, chaperone proteins in general recognize exposed hydrophobic surfaces of nonnative species, surfaces that will ultimately be buried in the native state, and form noncovalent interactions with them, stabilizing them against irreversible multimeric aggregation. Release of polypeptide then follows, in many cases driven by an ATP-directed conformational change of the chaperone, permitting subsequent steps of polypeptide folding or biogenesis to occur. When such steps fail to proceed productively, recognition and rebinding by the same or another chaperone can occur, allowing another opportunity for a productive conformation to be reached.

Different classes of molecular chaperones appear to be directed to binding specific nonnative states, the nature of which are beginning to be understood. For example, the two best-studied families, examined in detail below, the ubiquitous Hsp70 and Hsp60 (chaperonin) chaperones, recognize hydrophobic surface in the context of, respectively, extended and collapsed (globular) conformations, which are bound correspondingly either by local enclosure of the chain or by global enclosure of the polypeptide in a central cavity. Because there is not yet the detailed level of structural and mechanistic understanding for other recognized families of chaperones, they are not considered here, but important observations concerning binding, nucleotide use, and cellular actions are summarized in Table 1.

Hsp70 Chaperones—Activity Involves Cycles of Polypeptide Binding and Release

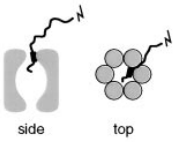
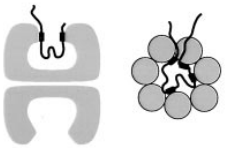
Hsp70 chaperones, with their co-chaperones, comprise a set of abundant cellular machines that assist a large variety of protein folding processes in almost all cellular compartments. Historically, they were identified by induction under conditions of stress, during which they are now known to provide an essential action of preventing aggregation and assisting refolding of misfolded proteins. But they also play an essential role under normal conditions, including (1) assisting folding of some newly translated proteins; (2) guiding translocating proteins across organellar membranes through action at both the *cis* and *trans* sides; (3) disassembling oligomeric protein structures; (4) facilitating proteolytic degradation of unstable proteins; and in selected cases, (5) controlling the biological activity of folded regulatory proteins, including transcription factors (for a discussion of these actions, see Morimoto et al., 1994; Hartl, 1996). All of these activities rely on the ATP-regulated association of Hsp70 with short hydrophobic segments in substrate polypeptides (Flynn et al., 1991; Rüdiger et al., 1997a), which prevents further folding or aggregation by shielding these segments.

In Hsp70-assisted folding reactions, substrates undergo repeated cycles of binding/release (Szabo et al., 1994; Buchberger et al., 1996), frequently at a stoichiometry of a single Hsp70 monomer per substrate molecule. Hsp70 binding does not appear to induce global conformational changes in the substrate but, rather, appears to act locally. Substrates released from the chaperone undergo kinetic partitioning between folding to native state, aggregation, rebinding to Hsp70, and binding to other chaperones or proteases as part of a multidirectional folding network. Hsp70 proteins all consist of the same working parts: a highly conserved NH_2-terminal ATPase domain of 44 kDa and a COOH-terminal region of 25 kDa, divided into a conserved substrate binding domain of 15 kDa and a less-conserved immediate COOH-terminal domain of 10 kDa (Figure 1).

Structure of an Hsp70 Peptide Binding Domain, and the Consensus Motif Recognized in Substrate Proteins

The molecular basis for Hsp70 binding to many nonnative proteins has been elucidated for the bacterial cytoplasmic homolog, DnaK, through biochemical and crystallographic studies. Studies with peptides have indicated that DnaK binds with greatest affinity to short hydrophobic segments in extended conformation (Schmid et al., 1994; Zhu et al., 1996). To qualify as a substrate, it thus seems a minimal requirement that a protein expose a single recognizable segment, either through local unfolding or as an intrinsically unfolded structural element, such as a loop. The crystal structure of the COOH-terminal substrate binding domain of DnaK complexed with a heptapeptide substrate, NRLLLTG (Zhu et al., 1996), reveals a β sandwich composed of two sheets of four strands each, followed in the primary structure by two α helices, A and B, which span back over the sandwich (Figure 2A). The top sheet emanates four loops, two of which ($L_{1,2}$–$L_{3,4}$) form the substrate binding pocket, a channel with a cross section of $\sim 5 \times 7$ Å. Along with loops flanking them at either side, they are stabilized by critical contacts with the overlying helix B, which may function as a lid in permitting entry and release of substrate (without directly contacting it) (Figures 2A and 2B). The peptide in the substrate binding pocket pierces the narrow dimension of the domain and is contacted by DnaK only through its central five residues (Figures 2C and 2D). The most extensive contacts are hydrophobic side-chain contacts between the loops and

Table 1. Topology of Polypeptide Binding and Action of Chaperone Families

CHAPERONE	TOPOLOGY OF BINDING	ACTION	REFERENCES
Hsp100		ATP-dependent disaggregation and unfolding for degradation	Schirmer et al., 1996; Levchenko et al., 1997
Hsp90	multiprotein complex	Conformational maturation of steroid hormone receptors and signal transducing kinases	Bohen et al., 1996; Prodromou et al., 1997
Hsp70 (DnaK)		ATP-dependent stabilization of hydrophobic regions in extended polypeptide segments	see text
Hsp60 (GroEL)		ATP-dependent facilitation of folding to the native state	see text
Small Hsps (Hsp25, etc.)		Stabilization against aggregation during heat-shock	Lee et al., 1997; Ehrnsperger et al., 1997
Calnexin/Calreticulin	?	Folding of glucosylated proteins in the ER in cooperation with glucosyltransferase	Sousa and Parodi, 1995; Helenius et al., 1997

Bold lines signify polypeptides, and the thickened segments denote sites that become directly associated with chaperone, typically hydrophobic in character. Structures are not drawn to scale.

the three central leucines in the peptide (L3–5, Figure 2D). The central-most leucine side chain resides in a hydrophobic pocket in the floor of the channel and is surmounted by a hydrophobic "arch" that also interacts with the hydrophobic side chains of the neighboring leucine residues in the peptide. Seven hydrogen bonds are also observed between the peptide and DnaK, formed between the main chain of the peptide and, in most cases, the main chain of DnaK. These contacts, coupled with the hydrophobic specificity-determining pockets, dictate the requirement for an extended conformation of the bound peptide. The interactive surface of DnaK at the ends of the hydrophobic channel is negatively charged and favors the presence of basic residues at the end positions of the peptide (e.g., arginine at position 2). More globally, binding/enclosure of the extended peptide appears to require that the interacting polypeptide segment be separated from the remainder of the substrate protein by 10 Å or more, implying that the bound region of the polypeptide must be substantially unfolded.

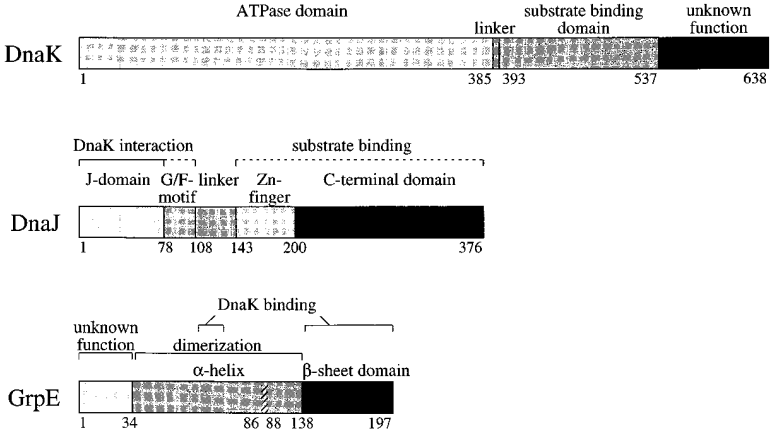


Figure 1. Domain Organization of DnaK, DnaJ, and GrpE

Individual domains of DnaK, DnaJ, and GrpE, residue numbers defining the approximate domain borders, known structural features, and functions of domains. The definition of domains is based on 3D-structures and sequence alignments using standard algorithms. DnaK: residues 386–392 constitute a linker between the ATPase and the substrate binding domain. GrpE: residues 86–88 constitute a break of the long NH_2-terminal α helix in the GrpE monomer that interacts with DnaK.

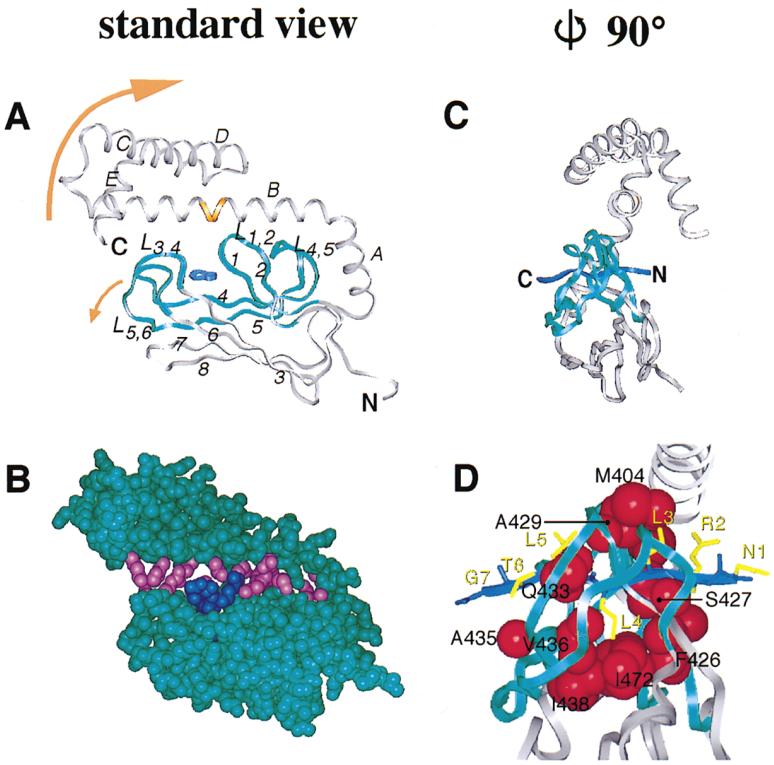


Figure 2. Substrate Binding Domain of DnaK in Complex with a Peptide Substrate

Ribbon diagram and space-filling models of the substrate binding domain (Zhu et al., 1996) in standard view (A and B) and views rotated 90° counterclockwise around the vertical axis of the standard view (C and D). In (A), the peptide substrate is shown in blue, the strands (1, 2, 4, and 5) and loops ($L_{1,2}$, $L_{3,4}$, $L_{4,5}$, and $L_{5,6}$) of DnaK constituting the upper β sheet in green, and the hinge-forming residues Arg^{536}–Gln^{538} of helix B, which may allow ATP-dependent opening of the helical lid (arrows in [A]), in orange. Helices, strands, and loops are labeled; the large N and C indicate the NH_2- and COOH-termini of the DnaK fragment. (B) shows the buried nature of the bound peptide substrate in space-filling representation: peptide in blue, DnaK in green, and side chains contributing to the interaction of helix B with the loops of the upper β sheet in pink. (C) is the rotated version of (A), colored similarly; here, N and C refer to the NH_2 and COOH termini, respectively, of the bound peptide. (D) shows details of the peptide binding cavity of DnaK: peptide backbone in blue, side chains of the peptide as yellow sticks, DnaK backbone in interacting region in green, and side chains of DnaK residues interacting with the peptide as red van der Waals spheres. All representations were produced using INSIGHT II, Biosym. (Modified from Rüdiger et al., 1997a).

A consensus motif recognized by DnaK in substrate polypeptides has been identified by screening a library of peptides derived from known protein sequences (Rüdiger et al., 1997b) and comprises a hydrophobic core of 4–5 residues flanked by basic residues. The hydrophobic cores of individual peptides recognized by DnaK contain typically 2–4 hydrophobic residues, with Leu the most common, present in ~90% of recognized peptides. Acidic residues are excluded from the cores and disfavored in the flanking regions. Such a motif occurs frequently within protein sequences, every 36 residues on average, and localizes preferentially to buried β strands of the corresponding folded proteins. The motif identified by this experimental approach corresponds remarkably well with the observed features of interaction of the NRLLLTG peptide from the structural study.

The ATPase Cycle Controls Substrate Binding by Hsp70 Proteins

ATP, bound by the NH_2-terminal domain of Hsp70, is used to drive conformational changes in the COOH-terminal peptide binding domain that alter its affinity for substrates (Figure 3). The binding of ATP increases the dissociation constant for Hsp70–substrate complexes between 5- and 85-fold as a result of increases in k_{off} of 2–3 orders of magnitude, coupled with increases in k_{on} of ~50-fold (Palleros et al., 1993; Schmid et al., 1994; McCarty et al., 1995; Theyssen et al. 1996; Pierpaoli et al., 1997). The ATPase cycle of Hsp70 can thus be viewed, in its simplest form, as an alternation between two states: the ATP-bound state, with low affinity and fast exchange rates for substrates (substrate binding pocket open), and the ADP-bound state, with high affinity and slow exchange rates for substrates (substrate binding pocket closed).

From the kinetic parameters of these two states, it is clear that the rapid association of Hsp70 with substrates can only occur in the ATP state, because substrate binding to the ADP state is too slow on the time scale of folding reactions. Dissection of ATP binding reveals that it occurs in two steps: first, the rapid formation of a weak complex, followed by a slower structural rearrangement (Ha and McKay, 1995; Theyssen et al., 1996), leading to an overall K_d for ATP in the submicromolar range (Gao et al., 1994; Ha and McKay, 1994; Theyssen et al., 1996). The second step probably reflects the precise locking-in of the nucleotide in the binding pocket, essential for hydrolysis (see below), and is kinetically coupled to the release or exchange of a previously bound polypeptide. The subsequent conversion of the ATP–peptide–Hsp70 ternary complex to the ADP state then stabilizes the chaperone–peptide interaction. The importance of this step has been demonstrated by the finding that mutant Hsp70 proteins, arrested in the ATP-bound state due to defects in hydrolysis, are completely deficient in chaperone activities (Ha et al, 1997).

Hydrolysis of ATP is the rate-limiting step in the ATPase cycle of Hsp70 proteins in isolation (Gao et al., 1993; McCarty et al., 1995; Karzai and McMacken, 1996; Theyssen et al., 1996) and likely results in dramatic conformational changes in Hsp70 that convert it to the high affinity, slow exchange state, which sequesters substrate protein. The final step in the ATPase cycle, the release of ADP and P_i, does not induce detectable conformational changes but allows the subsequent rapid

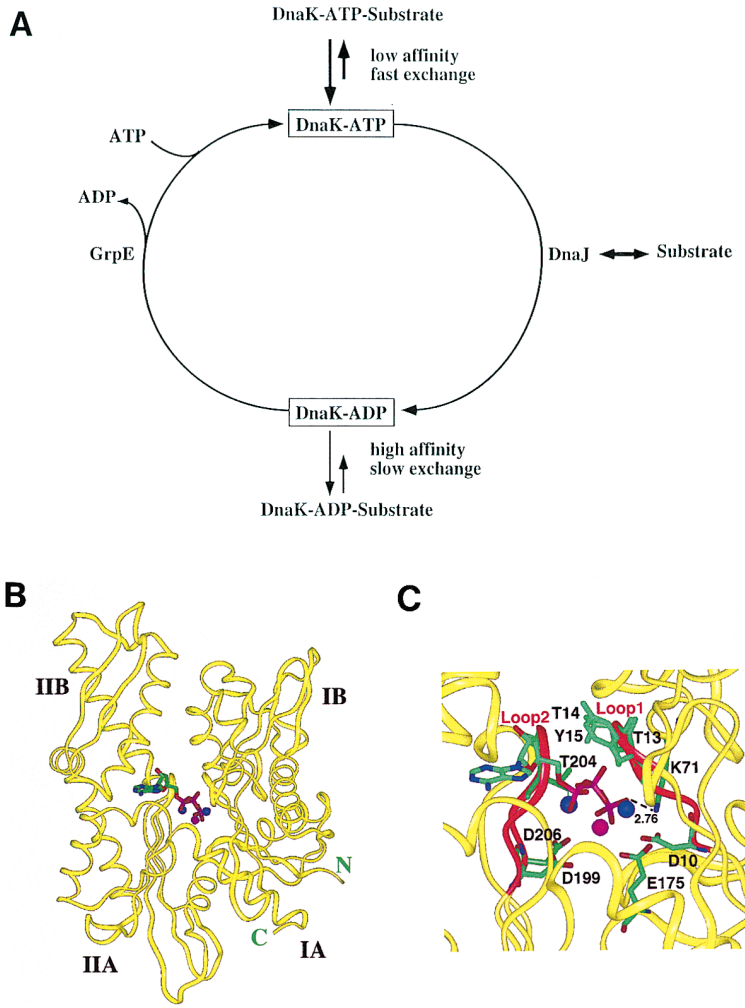


Figure 3. ATPase Cycle and Atomic Structure of the ATPase Domain of Hsp70 Proteins

(A) Schematic drawing of the regulated ATPase cycle of DnaK and its coupling to substrate binding.

(B and C) Structure of the ATPase domain of bovine Hsc70 (B). The ATPase domain structure (Flaherty et al., 1990) with indicated subdomains is shown with bound ADP, P_i, Mg^{2+} (magenta), and two K^+ ions (blue) indicated. (C) The nucleotide binding pocket of bovine Hsc70. The backbone of phosphate binding loops 1 (residues 10–15) and 2 (residues 199–206) is indicated in red, and the side chains involved in positioning the phosphates of bound nucleotide and Mg^{2+} (including Lys-71, implicated in catalysis of ATP hydrolysis) are shown as green sticks; nucleotide, Mg^{2+}, and K^+ are colored as in (B).

binding of ATP, which reestablishes the starting point. Although nucleotide exchange is 10–20-fold faster than the rate of hydrolysis in the unstimulated cycle (McCarty et al., 1995; Theyssen et al., 1996), it can become rate-limiting when the hydrolysis step is stimulated by co-chaperones (see below).

Structures of Hsp70 ATPase Domain

The nearly identical structures of the ATPase domains of DnaK and Hsc70 consist of two large, globular subdomains (I and II), separated by a deep central cleft and connected by two crossed α helices (Figures 3B and 3C). Both subdomains and the connecting helices contribute to forming the binding pocket for nucleotide and the required Mg^{2+} and K^+ ions at the bottom of the cleft (Flaherty et al., 1990). The nucleotide is positioned in the active site by interactions with two phosphate-binding loops and a hydrophobic adenosine binding pocket (Flaherty et al., 1990), together with contacts with the Mg^{2+} ion, which is coordinated by several side chains of Hsc70. Based on structural studies of wild-type and mutant Hsc70 proteins in the presence of a variety of nucleotides and metals, McKay and coworkers have proposed a mechanism for ATP hydrolysis (Flaherty et al., 1994; O'Brien et al., 1996): structural rearrangement of Hsp70 during ATP binding leads to adjustment of the position of the γ-phosphate so that a bidentate complex is formed between the β- and γ-phosphate oxygens and Mg^{2+}, permitting an in-line attack by a water (or OH^-) that is hydrogen-bonded to Lys-71 (Figure 3C). Precise geometry of the nucleotide and the surrounding residues requires the correct positioning of the Mg^{2+} ion, established in part by the binding of two K^+ ions nearby. This accounts for the absolute requirement of K^+ for ATP hydrolysis and chaperone activity of Hsp70 proteins (Palleros et al., 1993).

Coupling between Hsp70 ATPase and Peptide Binding Domains

The molecular mechanism by which the chemical energy of ATP is used to perform mechanical work, that is, the opening and closing of the substrate binding pocket, is poorly understood. The available atomic structures of the Hsc70 ATPase domain do not indicate large-scale motions in response to either nucleotide binding or hydrolysis. Either there are subtle conformational changes in the ATPase domain that are amplified to produce dramatic changes in the rest of the chaperone or the crystallized ATPase domain fragments do not reflect conformational changes occurring in the full-length protein in response to the nucleotide. Supporting the latter possibility, biochemical demonstration of nucleotide-dependent conformational changes in Hsp70 proteins requires the physical connection of the NH_2-terminal

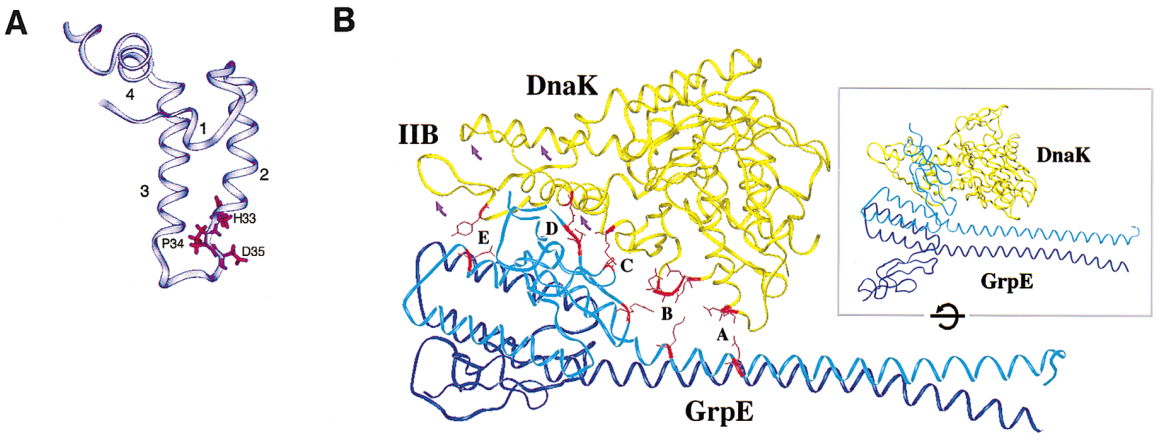


Figure 4. High-Resolution Structures of the J Domain and a Complex between GrpE and the ATPase Domain of DnaK

(A) The NMR structure of the J domain fragment of *E. coli* DnaJ (Pellecchia et al., 1996) is shown, with numbered helices and residues of the conserved HPD motif (red side chains) indicated. (B) The structure of a complex between NH_2 terminally truncated GrpE and the ATPase domain of DnaK (Harrison et al., 1997) is shown. The DnaK ATPase domain is in yellow, and the GrpE dimer (truncated by 33 residues) in dark blue (DnaK distal monomer) and light blue (DnaK proximal monomer), respectively. All contacts between GrpE and DnaK are mediated by the DnaK proximal monomer of GrpE. The inset is rotated by 90° about the horizontal axis to further illustrate the site of contact. Side chains of residues involved in major contacts between both molecules are shown in red, and sites of major contact are lettered. GrpE binding induces conformational changes in DnaK, indicated by the purple arrows, that lead to an opening of the nucleotide binding pocket.

ATPase domain with the adjacent substrate binding domain (Buchberger et al., 1995). In particular, several studies suggest that ATP binding triggers an association of the ATPase domain with the substrate binding domain and that this causes further conformational changes within the substrate binding domain itself (Buchberger et al., 1995; Ha and McKay, 1995; Wilbanks et al., 1995), although the precise changes that open the substrate binding pocket remain unknown.

Whatever the coupling mechanism, differences in the structure of the substrate binding domain in two crystal forms of DnaK have led Hendrickson and coworkers to propose a structural basis for the ATP-induced opening of the binding pocket (Zhu et al., 1996). One structure has a "kink" at residues 536–538 of the lid-forming α helix B (indicated by orange segment in Figure 2A), and consequently, the subdomain COOH-terminal to the substrate binding domain has rotated upwards by 11°, causing a loss of stabilizing contacts between α helix B and the outer loops. It has been proposed that this represents the initial stages of release by a "latch" mechanism and that further movement of the "lid" opens up the substrate binding pocket and triggers substrate release.

Control of the Hsp70 ATPase Cycle by Co-chaperones

The steady-state turnover rate of the unstimulated Hsp70 ATPase is too slow (between 0.02 and 0.2 min^{-1}) to drive the chaperone activities of Hsp70, even in the presence of substrates, which typically stimulate the ATPase activity 2–10-fold (Flynn et al., 1989; Gao et al., 1994; Ha and McKay, 1994; Jordan and McMacken, 1995; McCarty et al., 1995; Theyssen et al., 1996). Therefore, it is essential that regulatory mechanisms exist to increase ATP turnover and, hence, chaperone function (Figure 3A). ATP hydrolysis is the prime target for regulation, mainly by members of the DnaJ family (Liberek et al., 1991; McCarty et al., 1995), found in all Hsp70-containing compartments of prokaryotic and eukaryotic cells, as well as in several tumor viruses (Laufen et al., 1997). DnaJ proteins are a heterogeneous group of multidomain proteins defined by a highly conserved domain of ~80 amino acids, the J domain, often located near the NH_2 terminus, which is essential for stimulation of the Hsp70 ATPase activity (Figure 1; Wall et al., 1994; Karzai and McMacken, 1996; Szabo et al., 1996). Solution structures of the J domain from two family members (Szyperski et al., 1994; Pellecchia et al., 1996; Qian et al., 1996) show that it comprises four helices with a loop between helices 2 and 3 containing a conserved sequence motif (HPD) implicated in interaction of the J domain with Hsp70 (Figure 4A; Wall et al., 1994).

Regulation of release of ADP and P_i from Hsp70 is also essential for some homologs, such as bacterial DnaK and mitochondrial Ssc1p, and is accomplished by members of the GrpE family (Figure 1) (Liberek et al., 1991; Dekker and Pfanner, 1997; Miao et al., 1997). Association of GrpE with DnaK–ADP accelerates nucleotide exchange 5000-fold, reducing the affinity of DnaK for ADP 200-fold (Packschies et al., 1997). For the DnaK system, GrpE and DnaJ together stimulate the ATP turnover rate at least several hundred-fold at saturating conditions (McCarty et al., 1995), which may be more than is necessary to support chaperone function. The effects of DnaJ and GrpE have to be balanced to optimize the equilibrium between substrate binding and release; this is achieved in vivo by coregulation of expression of their genes.

A structure of the stable complex between a dimer of NH_2 terminally truncated GrpE and the ATPase domain of DnaK (Figure 4B) shows that GrpE triggers nucleotide exchange by a contact through one GrpE subunit that opens the nucleotide binding cleft of DnaK, as manifested by a 14° rotation (purple arrows in Figure 4B) of the IIB subdomain of the DnaK ATPase domain (relative to its position in the ADP-bound structure of the Hsc70 ATPase domain) (Harrison et al., 1997). This motion displaces DnaK residues (Ser-274, Lys-270, and Glu-267)

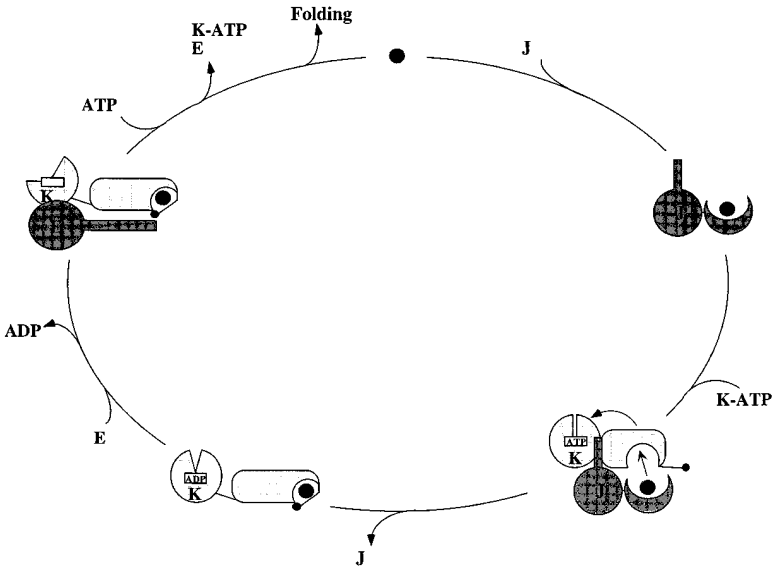


Figure 5. Model of the Chaperone Cycle of the DnaK System

The cycle starts with the association of DnaJ (J) with a substrate (closed circle), followed by transfer of the substate to the ATP form of DnaK (K). This transfer is coupled to the locking-in of the substrate in the substrate binding pocket of DnaK by ATP hydrolysis. Following substrate transfer, DnaJ leaves the complex, and GrpE (E) associates with the DnaK–substrate complex to trigger ADP release from DnaK. This allows binding of ATP and subsequent release of GrpE and substrate from DnaK.

that provide crucial hydrogen bonds to the adenine and ribose rings of bound ADP.

It is intriguing that GrpE homologs appear to be lacking in the cytosol, nucleus, and endoplasmic reticulum of eukaryotic cells. A nucleotide exchange factor thus seems dispensable for at least some chaperone activities of cytosolic Hsp70 homologs, such as Ssa1p of yeast (Levy et al., 1995) and Hsp70/Hsc70 of the mammalian cytosol (Freeman and Morimoto, 1996). For Ssa1p, it appears that its DnaJ co-chaperone, Ydj1p, stimulates not only ATP hydrolysis but also product release (Ziegelhoffer et al., 1995) and thus may have a dual regulatory role in the ATPase cycle. Further variations in regulation of the functional cycle of Hsp70 proteins may exist, given that other Hsc70/Hsp70 binding proteins recently identified in the eukaryotic cytosol are proposed to stabilize the ADP state (Hip) or stimulate nucleotide exchange (Hop, also known as p60 or Sti1; and BAG-1) (Frydman and Höhfeld, 1997; Höhfeld and Jentsch, 1997).

DnaJ-Mediated Coupling of ATPase with Substrate Binding: A Model for the Chaperone Cycle of Hsp70

The mechanism by which the action of DnaJ proteins couples the regulated ATPase cycle of Hsp70 with productive substrate binding is key to the entire functional cycle of Hsp70 proteins. Such coupling prevents stimulating futile ATPase cycles in the absence of substrate. In the case of the DnaJ–DnaK system, it has been observed that in addition to binding to DnaK, DnaJ itself associates with substrates of the DnaK system with kinetics fast enough to prevent their aggregation (Langer et al., 1992a; Schröder et al., 1993; Gamer et al., 1996), possibly by binding to a sequence motif similar to that recognized by DnaK. Furthermore, the efficiency with which DnaJ stimulates the ATPase activity of DnaK is strongly increased by the presence of polypeptide substrates. These data suggest a revised model of the functional cycle of the DnaK system (Figure 5): (1) The cycle starts with the transient and rapid association of DnaJ with substrates, although in some cases the cycle may start with the association of DnaK–ATP with a substrate. (2) DnaK–ATP accepts polypeptide from the DnaJ–substrate complex in a process requiring two steps, transient interaction of DnaK–ATP with the J domain of DnaJ through an undetermined DnaK binding site and transfer of substrate protein from DnaJ to the open substrate binding pocket of DnaK–ATP. Both steps together are required to stimulate ATP hydrolysis by DnaK, resulting in stabilization of the DnaK–substrate complex and tightly coupling ATP hydrolysis to substrate binding by DnaK. (3) Upon substrate transfer to DnaK and conversion of DnaK to the ADP state, the affinity of the DnaK–substrate complex for DnaJ is reduced, and DnaJ dissociates. This step is reflected in observations that ternary DnaK–DnaJ–substrate complexes are unstable (Gamer et al., 1996) and that DnaJ acts catalytically in targeting DnaK to substrates (Liberek et al., 1995). (4) GrpE binds to the DnaK–ADP–substrate complex, triggering the release of ADP. Consequently, (5) ATP binds rapidly to DnaK, which releases the bound substrate and GrpE and returns DnaK to its initial state.

The Chaperonins—Binding and Folding Proteins in a Central Channel

Among the group of cellular machines utilizing ATP binding and hydrolysis to drive ordered conformational changes, one of the least expected and most fascinating devices to be uncovered are the chaperonins—the collective of double-ring assemblies that promote folding of proteins to the native state. These complexes, weighing in at nearly a million daltons and composed of back-to-back rings of identical or closely related rotationally symmetric subunits (~60 kDa), play an essential role in all cells, assisting a large variety of newly synthesized and newly translocated proteins to reach their native forms by binding them and facilitating their folding inside a large central channel within each ring (for discussion of action in vivo, see Ellis, 1996; Fenton and Horwich, 1997). The central cavity of each ring, the worksite of the machine, functions in two major states. In the binding-active state, it is open at the end of the

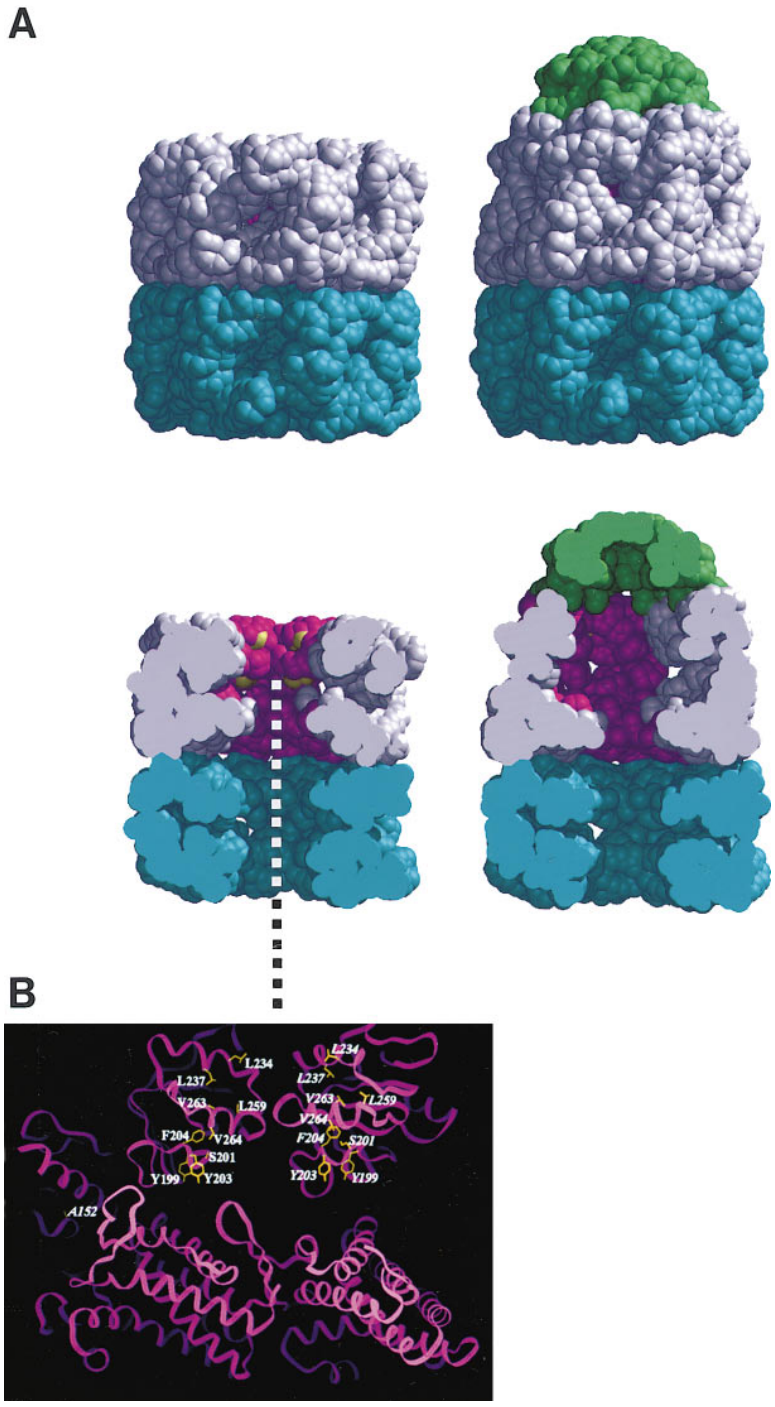


Figure 6. Architecture of GroEL and GroEL–GroES–$(ADP)_7$ Complexes and the Apical Polypeptide Binding Sites

(A) Space-filling models (6 Å Van der Waals spheres around Cα) of GroEL (left) and GroEL–GroES–$(ADP)_7$ (right). The upper panels are views from outside; the lower panels are from the inside, generated by slicing the models with a vertical plane that contains the cylindrical axis. The lower GroEL ring is blue; GroES is green; and two subunits of the upper ring are colored magenta (main chain) and yellow (side chains), corresponding to the subunits in (B). (B) Ribbon diagram of two neighboring subunits from the top ring, showing location of apical domain residues involved in polypeptide binding: main chain in magenta, with yellow sticks indicating the side chains of the residues implicated by mutational analysis in polypeptide binding (and GroES binding).

cylinder for ingress of nonnative proteins (Langer et al., 1992b; Braig et al., 1993), exposing a flexible hydrophobic lining that likely binds nonnative species through exposed hydrophobic surfaces (which will become buried to the interior in the native state) (Figure 6) (Braig et al., 1994; Fenton et al., 1994). Binding is most likely multivalent in character, with the substrate protein contacted simultaneously by many of the chaperonin apical (end) domains surrounding the channel. For some proteins, such binding may be associated with an action of partial unfolding, serving to "unscramble" a misfolded state and, in energetic terms, removing the protein from a kinetic trap (e.g., Ranson et al., 1995; Zahn et al., 1996). Regardless of how it becomes bound, a captured nonnative protein is conferred the extraordinary opportunity to reach the native state after release from the binding sites when the machine proceeds to its other state, the folding-active state.

The folding-active state is reached by conformational changes in the chaperonin, induced by the action of ATP binding and, for the organellar/bacterial chaperonins, by binding also of a lid-like co-chaperonin, itself a ring of

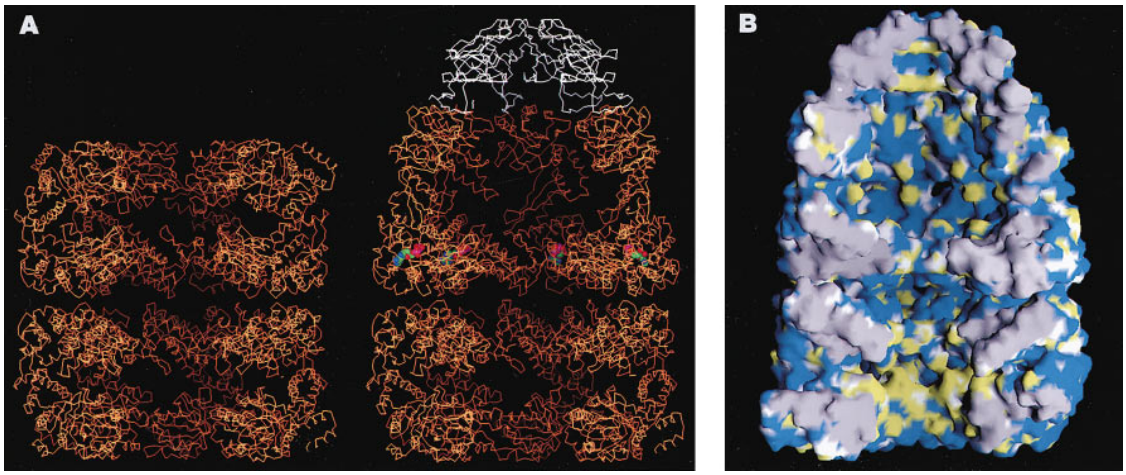


Figure 7. Views of the Central Cavity and Polypeptide Binding Surfaces of GroEL

(A) Cα skeleton drawings of GroEL (left) and GroEL–GroES–$(ADP)_7$ (right), sliced vertically along the central axis. Note that the interaction of GroES (white) with GroEL (orange) forms a continuous dome-shaped cavity of 2-fold increased volume relative to the unliganded structure. The nucleotide (colored balls) is shown in its binding sites in the right panel.

(B) An interior view of four subunits from each ring of the asymmetric structure, colored to reflect the relative hydrophobicity of the interior surface. Hydrophobic side-chain atoms are yellow; polar and charged side-chain atoms are blue; solvent-excluded surfaces at the interfaces with the missing subunits are gray; and exposed backbone atoms are white.

rotationally symmetric subunits (each of ~10 kDa) (Hunt et al., 1996; Mande et al., 1996) that trigger folding of the substrate protein encapsulated in the central channel (Weissman et al., 1995; Mayhew et al., 1996; Weissman et al., 1996). The conformational change of the bacterial chaperonin, GroEL, upon binding its co-chaperonin, GroES, initially detected by EM (Chen et al., 1994; Roseman et al., 1996) and recently resolved crystallographically at 3 Å resolution (Xu et al., 1997), is a molecular spectacle, with dramatic en bloc movements of the seven apical domains in the ring bound by nucleotide and co-chaperonin, resulting in a global change of the shape and character of the central cavity. The cavity of the bound ring enlarges 2-fold in volume and is closed off at the open end by the dome-shaped GroES ring, encapsulating/sequestering the nonnative protein (Figure 7A); the hydrophobic binding surface is elevated and twisted away from the polypeptide, releasing it into the cavity; and the aspect of the apical domains now forming the cavity surface is hydrophilic in character (Figure 7B), favoring burial of hydrophobic surfaces in the folding substrate protein and exposure of its hydrophilic surface, thus acting to promote the native state (Xu et al., 1997).

Because chaperonins thus appear to function in significant measure through the alternate temporal exposure of surface hydrophobicity and hydrophilicity, it seems comprehensible how they can bind and productively release a large variety of different nonnative proteins that do not share any common primary or secondary structural property. As such, the machines do not appear to convey any steric information. This, rather, is provided by the primary structure of the substrate proteins, as recognized early by Anfinsen and coworkers. Chaperonins, on the other hand, provide kinetic assistance to the folding process which, under in vivo conditions, is liable to nonproductive steps that can lead to misfolded states that lie in local energetic minima, so-called kinetic "traps" (taking the native state as typically lying at a global minimum). Energy is required to unscramble such conformations, allowing conversion (over energy barriers) to other conformations that are more energetically favored to reach the native state. Theoretically, there are myriad ways in which a polypeptide can misfold (Dill and Chan, 1997), but there may be only a limited number of off-pathway steps that can deter correct folding of a given polypeptide (e.g., Yeh et al., 1997). Although the exact conformation of any misfolded form recognized by a chaperonin remains to be precisely determined, it seems likely that substantial amounts of native-like secondary and tertiary structure are present in unstable collapsed structures. Such species studied in isolation generally expose hydrophobic surfaces that are susceptible to multimeric aggregation. Such a fate is forestalled by competing interactions with the hydrophobic surface lining the channel of a chaperonin, which stabilizes against aggregation. Capture on this surface then commits the substrate to an opportunity to fold productively in the favorable environment produced when the machine switches to the folding-active state.

The Working Parts of Chaperonin

To enable the large domain movements that switch the central cavity of a chaperonin ring between peptide-accepting and folding-active states, the apical domains are each hinged to the top of a slender intermediate domain that is in turn hinged at its lower aspect to a relatively fixed base, the equatorial domain, which houses the ATP binding site (Figure 8) (Braig et al., 1994; Boisvert et al., 1996). In the transition from binding-active to folding-active conformation, there are en bloc movements about both of these hinges, a 25° downward rotation about the lower point, bringing the intermediate domain down onto the equatorial domain, locking the

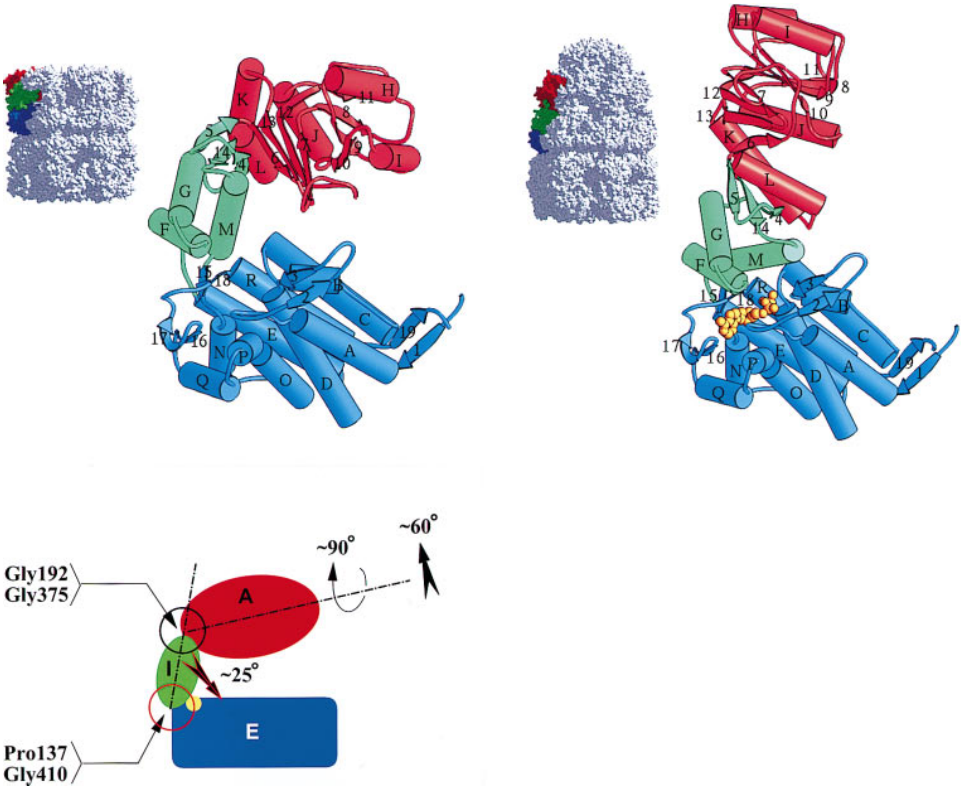


Figure 8. The Direction and Magnitude of the en bloc Domain Movements within an Individual Subunit of the *cis* GroEL Ring Accompanying Binding of ATP and GroES

The upper panels show ribbon diagrams of an individual subunit of unliganded (left) and liganded (right) GroEL, oriented with the 7-fold axis to the right, as indicated in the space-filling models (insets). Note that GroES is not shown in the right-hand panel, to reveal more clearly the extent of motion of the apical domain. The equatorial, intermediate, and apical domains are blue, green, and red, respectively. The nucleotide (ADP) in the right-hand structure is a yellow space-filling model. The lower panel shows diagrammatically the en bloc movements that occur around the pivot points at the ends of the intermediate domain. Domains are colored as in the upper panels, and the small yellow circle on the top of the equatorial domain represents the nucleotide.

nucleotide into the ATP site, and a 65° rotation upward about the upper hinge, permitting a dramatic upward elevation of the apical domain that is accompanied by a 90° clockwise twist of the domain about its long axis (Figure 8, lower panel) (Xu et al., 1997). The movements of the apical domain thus translocate the hydrophobic aspect that originally faced the cavity up and 90° away, to a position where one portion of the hydrophobic surface makes contact with GroES, while the remaining portion contributes to a new interface between the mobilized apical domains. The contact with GroES is mediated through mobile β-hairpin loop segments (Landry et al., 1993; Hunt et al., 1996), one from each GroES subunit, that extend downward and laterally to make contact with a corresponding GroEL apical domain (Xu et al., 1997). The contact is in part hydrophobic in character, involving interaction between several of the GroEL apical hydrophobic residues required for polypeptide binding (Fenton et al., 1994) and a sequence, Ile-Val-Leu, in one "edge" of the GroES mobile loop. The new interface formed between apical domains involves contact between the mobilized hydrophobic surface of one subunit and the back aspect (formerly the outside surface) of the neighboring subunit. Both the seven-valent, subunit-to-subunit contacts between GroES and GroEL and the new supporting interfaces act to stabilize the opened-up, folding-active conformation of the apical domains (Xu et al., 1997). Although nucleotide binding alone, in the absence of GroES, has not been observed to produce this extent of apical movement (Boisvert et al., 1996; Roseman et al., 1996), it must be capable of transiently driving the full or nearly full extent of these changes, which are then stabilized by GroES binding.

In the same way that nucleotide binding promotes the opening of the apical domains, enabling GroES binding and stabilization of the folding-active state, GroES binding stabilizes the nucleotide-bound conformation by maintaining the intermediate domain in the conformation that locks the nucleotide into the equatorial site (Figure 8) (Xu et al., 1997). In particular, when the favored nucleotide, ATP, is bound in this manner, it becomes committed to hydrolysis in a "quantized" fashion, with the GroES-bound ring turning over a packet of 7 ATPs, one in each of the seven subunits (Todd et al., 1994). Such committed hydrolysis appears to result from more than simple enclosure of ATP in the active site—the locked-down intermediate domain also contributes an aspartate side chain (D398) that enters directly into the nucleotide pocket distal to the β phosphate, contributing to coordination of a Mg^{+2} in the site (Xu et al., 1997).

Mutational alteration of this residue (to Ala) reduces ATP hydrolysis to ~2% of wild type, without affecting the affinity for ATP (or for GroES) (Rye et al., 1997). It thus seems that the intermediate domain contributes directly to the ATPase machinery. Even with these structural insights, the mechanism of ATP hydrolysis by GroEL remains to be resolved.

The structure of a binary complex formed with a transition-state nucleotide analog, or one containing ATP in the setting of a mutation preventing hydrolysis, might be revealing in this regard. In addition, such a structure might provide information about the long-range effects of the γ-phosphate on the elevation and twist of the apical domains as compared with the ADP–GroES structure. This is significant with respect to the activation of productive folding, insofar as only ATP, in the presence of GroES, can promote full release of stringent substrate proteins into the channel, followed by productive folding (Rye et al., 1997). Other nucleotides, including ADP and AMP-PNP, fail to promote release of such substrates. CryoEM image reconstruction studies of GroEL–GroES complexes have been interpreted to suggest that there may be a more extreme clockwise twist of the apical domains in ATP as compared with the other nucleotides (Roseman et al., 1996), indicating that the presence of the natural γ-phosphate drives additional crucial structural changes in the apical domains. The γ-phosphate also must play a critical but yet-to-be understood role in stabilizing the GroEL–GroES complex, because GroEL–GroES–ATP complexes are much more stable than corresponding GroEL–GroES–ADP ones (Rye et al., 1997), possibly related to putative additional clockwise twist produced by ATP. Thus, the state of GroEL–GroES that triggers polypeptide folding, the ATP state, is also the state with high affinity for GroES.

While the dramatic workings just described are proceeding in one GroEL ring, the opposite ring is otherwise engaged, occupying a conformation resembling that of unliganded GroEL, with its apical domains in the "down" position, as if to accept the polypeptide (Figure 7A). This reflects that the machine, although appearing in its unliganded state as two symmetric rings, becomes asymmetric upon exposure to its nucleotide and GroES ligands and, as a consequence, behaves asymmetrically with respect to a polypeptide ligand as well. For example, ATP binding to one GroEL ring is cooperative in nature (Gray and Fersht, 1991; Bochkareva et al., 1992; Jackson et al., 1993) but exerts an anticooperative action on ATP binding in the opposite ring (Bochkareva and Girshovich, 1994; Yifrach and Horovitz, 1995). Likewise, GroES binding to one ring (requiring the presence of nucleotide and associated with increased cooperativity of ATP hydrolysis) exerts a negatively cooperative action on binding of a second GroES to the opposite ring. A structural basis to this latter behavior is apparent from the GroEL–GroES–$(ADP)_7$ structure, where the subunits of the ring with bound GroES exhibit a small tilt of 4° inward toward the cylinder axis at the GroES end and, correspondingly, an outward tilt at the equatorial aspect. Because the structural details of the equatorial domains and ring–ring interface are preserved, the opposite, so-called *trans* ring is necessarily tilted outward, by ~2°, competing against a second GroES binding in *trans*. Correspondingly, with GroES bound only to the *cis* ring, the nucleotide is "locked" only into the seven *cis* nucleotide sites, while the sites in the *trans* ring are unoccupied (Figure 8) (Xu et al., 1997).

Action on the polypeptide substrate is necessarily affected by the asymmetric binding of nucleotide and GroES. The *trans* ring of the folding-active complex remains accessible to nonnative polypeptides in its open cavity, as revealed by the GroEL–GroES–$(ADP)_7$ structure (Figure 7) and observed biochemically (Weissman et al., 1995). Yet, it remains unclear whether such *trans* ternary complexes can directly become productive *cis* complexes by binding ATP and a second GroES molecule on the *trans* ring (while simultaneously discharging the *cis* ring) (see below). Nevertheless, it seems that only one GroEL ring at a time can occupy a folding-active state. Such asymmetry of the rings, as discussed below, appears necessary to allow an ordered progression of the machine through its reaction cycle.

Actions of ATP Binding and Hydrolysis in Driving GroEL Chaperonin through Its Conformational States

Given the asymmetry of the chaperonin machine in the presence of its ligands, it has seemed likely that ATP would have distinct actions in *cis* (GroES-bound) and *trans* rings. These roles in the formation and dissolution of a folding-active *cis* complex (Figure 9) have been resolved through biochemical studies, including single ATP turnover experiments and the analysis of single ring mutants and mutant rings able to bind but not hydrolyze ATP. It was observed (Todd et al., 1994; Hayer-Hartl et al., 1995) that GroES and bound *cis* ADP were rapidly discharged upon exposure of such asymmetric complexes to ATP, indicating that ATP binding or hydrolysis in the *trans* ring sends an allosteric signal that evicts the ligands from the *cis* ring (Figure 9, panel 6). Consistent with this interpretation, a single-ring version of GroEL bound GroES in the presence of ATP but could not release GroES or refolded protein, apparently resulting from the failure to receive a signal from the nonexistent *trans* ring (Weissman et al., 1995, 1996).

While the requirement for ATP in the *trans* ring was recognized early, a specific requirement for ATP in the *cis* ring has only recently been uncovered through observations that single-ring, obligatorily *cis* versions of GroEL can productively fold "stringent" substrates, such as RUBISCO from *Rhodospirillum rubrum* or mitochondrial malate dehydrogenase (MDH), in GroES and ATP (Rye et al., 1997). In addition, earlier kinetic observations that ATP is the preferred nucleotide for GroES binding (Jackson et al., 1993) suggest that, under physiologic conditions, the majority of GroEL-bound protein is triggered to commence folding by formation of GroES-ATP *cis* ternary complexes (Figure 9, panel 4).

It was unclear whether *cis* folding was triggered by ATP-GroES binding or by subsequent *cis* ATP hydrolysis. This was resolved by study of a mutant single ring complex bearing the intermediate domain substitution, D398A, that reduces ATP hydrolysis to ~2% of wild type. Conversion of bound substrate polypeptide to the native state occurred inside the D398A–GroES complex only in the presence of ATP (and not AMP-PNP), with the same kinetics and to the same extent as in hydrolysis-competent single and double rings, despite the absence

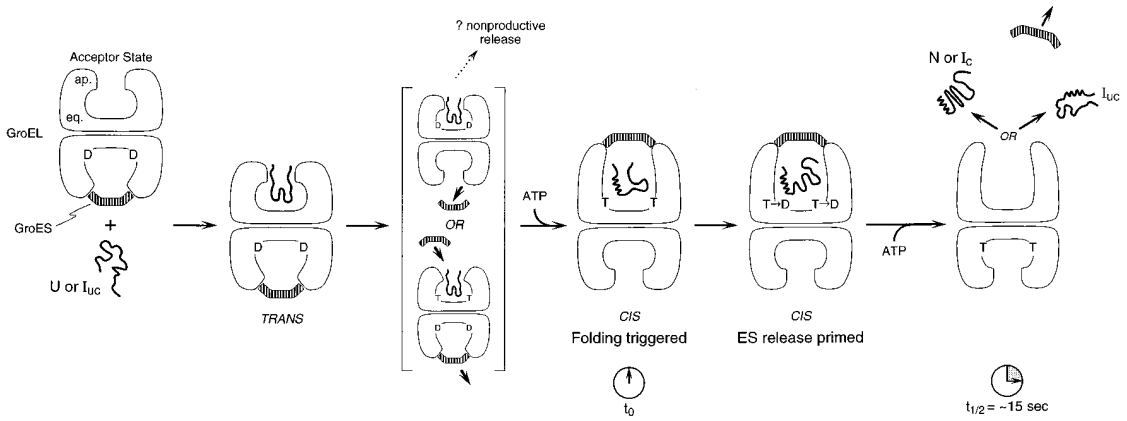


Figure 9. Model for a GroEL–GroES-Mediated Folding Reaction

The asymmetric GroEL–GroES complex (first panel; apical domain, ap.; equatorial domain, eq.) is the likely polypeptide acceptor state in vivo and binds unfolded polypeptides (U) or kinetically trapped folding intermediates (I_{uc}) to form a *trans* ternary complex (second panel). This complex is highly dynamic with respect to GroES binding in the presence of ATP (third panel); two possible pathways of GroES rearrangement that lead to the *cis* complex are shown. When GroES binds to the ring containing polypeptide in the presence of ATP (forming the folding-active *cis* intermediate, fourth panel), major conformational changes occur in the *cis* GroEL ring, similar or idenical to those shown in Figure 8, polypeptide release from the apical binding sites is triggered, and folding commences (t_0). Hydrolysis of ATP in the *cis* ring weakens the GroEL–GroES interaction (fifth panel), priming GroES release, while polypeptide folding continues. Binding of ATP in the *trans* ring evicts GroES from the *cis* ring (last panel), giving polypeptide the opportunity to depart ($\hat{t}$ = ~15 s). The released polypeptide is either native (N) or committed to fold (I_c), or is in an uncommitted or kinetically trapped state (I_{uc}), which can rebind to the same or a different GroEL complex and undergo another cycle of folding. In the complexes, D designates ADP; T, ATP; and T→D, ATP hydrolysis.

of ATP hydrolysis on this time-scale (Rye et al., 1997). Thus, ATP-GroES binding, and not hydrolysis, is required to produce the native state.

Remarkably, folding-active *cis* ATP–GroES–GroEL is a very stable complex that even resists dissociation by chaotrope. Thus, the energy of the γ-phosphate and its contacts are employed to assure the stability of the folding-active environment. Paradoxically, however, this complex is so stable that the refolded substrate remains locked up inside of it. It seemed likely that hydrolysis of *cis* ATP was the next step forward, based on the hypothesis that it would relax the high affinity interaction between GroES and GroEL (Figure 9, panel 5). This was supported by observation of the reduced stability of de novo–formed *cis* ADP versus ATP complexes and the lability of stable ATP complex attendant upon hydrolysis of *cis* ATP (Rye et al., 1997). Thus, hydrolysis of *cis* ATP acts to "prime" the *cis* ring for releasing GroES upon receiving the eviction signal sent by ATP in the *trans* ring (Figure 9, panels 5 and 6).

The observation that binding of ATP/GroES, but not AMP-PNP/GroES, triggered folding in the *cis* ring raised the issue of whether it is likewise ATP binding acting in *trans* that evicts the *cis* ligands (Figure 9, panel 6). Once again, the hydrolysis-defective D398A ring was used, and binding of ATP, but not AMP-PNP, to such a *trans* ring evicted the *cis* ligands (Rye et al., 1997).

Thus, binding of the nucleotide and not hydrolysis triggers the major work on the substrate protein: *cis*-bound ATP/GroES triggers release of substrate into the cavity followed by productive folding; *trans*-bound ATP triggers discharge of GroES and the substrate protein (Figure 9). Hydrolysis steps are used to drive the machine, directionally, to the next state. *Cis* hydrolysis relaxes the high affinity interaction of bound GroES for GroEL, "priming" the *cis* ring for release of GroES and substrate protein, but the precise action of *trans* hydrolysis is less clear at this point. One possibility is that ATP binding in *trans* may enable a second GroES molecule to bind to GroEL. If a polypeptide has been bound in the *trans* ring, then this would, at the same time as discharging the *cis* complex on the opposite ring, produce a new *cis* ternary complex (e.g., see Sparrer and Buchner, 1997). Such a step would reflect a "two-stroke" action of the GroEL machine, and ATP hydrolysis would always occur in the context of a *cis* complex, proceeding alternately on one ring, then the other (Lorimer, 1997). While such a model seems economical, several results argue against it. In one experiment, *trans* ternary complexes were formed in ADP with GroES and a substrate protein, ornithine transcarbamylase (OTC). Following addition of ATP and additional GroES, as well as a "trap" mutant of GroEL, able to bind but not release nonnative substrate, no OTC enzymatic activity was recovered (W. Fenton, personal communication). This implies that, instead of GroES binding to the OTC-containing ring and forming a productive *cis* complex, OTC was released and trapped. In a second experiment, the kinetics of folding by a mixed-ring GroEL complex, able to bind substrate and GroES on only one of its rings, was measured and found to be the same as wild-type GroEL (Burston et al., 1996). Here, if GroEL were functioning through a two-stroke mechanism, the wild-type would have been expected to produce more rapid recovery of activity than the mixed-ring complex.

The timing of the sequence of binding and hydrolysis of ATP in the *cis* and *trans* GroEL rings has obvious influence on the fate of substrate polypeptide (Figure 9). At 23°C, polypeptide is released into the central channel within a second of the binding of ATP and GroES to

form a *cis* complex (Rye et al., 1997). The substrate then has ~20 s in this favorable environment (Todd et al., 1994; Ranson et al., 1997): ~5–10 s in an ATP-*cis* state (Burston et al., 1995) and, after *cis* hydrolysis, ~10 s in an ADP state, following which GroES is discharged by binding of ATP in the *trans* ring. From dynamic measurements of substrate fluorescence as well as activity, it seems that there is a seamless transition of the folding reaction between *cis*-ATP and ADP, with no discernible phases, even though in structural terms, the apical domains are likely to occupy different conformations. Thus, while the initiation of folding of stringent substrates cannot be triggered by ADP–GroES, the progression of folding, once release has been driven by ATP–GroES binding, does not appear to be affected by conversion to the ADP state. A lifetime for the folding-active state of ~20 s may be sufficient for many substrates to reach the native state, but for others, only a fraction of the molecules reach native form before the "timer" goes off and GroES is discharged from the *cis* ring, ending the lifetime of the favorable environment. Along with GroES departure, both native and nonnative molecules have been observed to leave the *cis* cavity (Figure 9) (Burston et al., 1996).

Action of GroEL Chaperonin on Polypeptide

While the conformational states and dynamics of the GroEL machine itself are emerging at high resolution, the conformational states of substrate polypeptide during binding and folding are much less resolved. A number of questions are important to answer: Is polypeptide partially unfolded upon binding to GroEL? Is polypeptide further unfolded upon *cis* complex formation, stretched on the binding sites as they twist away from the central channel? Following release from the binding sites, is there any further interaction with the channel walls? Or is folding in the channel essentially a reaction carried out at infinite dilution? Finally, does rebinding to chaperonin take the substrate back to an "original" state, or is there progression of conformation toward the native state during multiple rounds of interaction of substrate with GroEL? Data bearing on a few of these points are summarized below, and they are considered in more detail in a recent review (Fenton and Horwich, 1997).

Polypeptide Binding. Early studies of GroEL action in vitro revealed that it could capture transient conformations that otherwise irreversibly aggregated (Goloubinoff et al., 1989), likely by stabilizing the exposed hydrophobic surface (Mendoza et al., 1991). More recent calorimetry studies revealed a negative heat capacity change on binding subtilisin to GroEL, indicative of hydrophobic interaction (Lin et al., 1995). Mutational analysis of GroEL revealed that the residues required for polypeptide binding are hydrophobic in character, localizing to the surface of the apical domains facing the central cavity (Braig et al., 1994; Fenton et al., 1994). Recently, a direct interaction was observed in a crystal of the isolated monomeric GroEL apical domain between residues of an NH_2-terminal tag segment and the apical binding surface of a neighboring monomer in the crystal lattice (Buckle et al., 1997), involving mostly nonpolar contacts between hydrophobic side chains of the tag segment and those of the binding site, with a few hydrogen bonds also formed between the main chain of the tag and several hydrophilic side chains at the binding surface.

The overall structural context in which hydrophobicity is recognized has been found to be, in general, a collapsed but loosely packed conformation, containing native-like secondary structure and, in some cases, a global topology that may be native-like. GroEL binds polypeptides within 10–100 ms after initiation of refolding (Katsumata et al., 1996; Goldberg et al., 1997; see also Murai et al., 1995; Ranson et al., 1997), when they have undergone collapse, and much of their native secondary structure is present. Tryptophan fluorescence studies (Martin et al., 1991; Mendoza et al., 1992) and hydrogen-deuterium exchange experiments (Robinson et al., 1994; Gervasoni et al., 1996; Groβ et al., 1996; Goldberg et al., 1997; but see also Zahn et al., 1994) have also indicated the presence of partial structure in bound polypeptides, even, in the case of DHFR, implying that a native-like global topology is present in the GroEL-bound state (Goldberg et al., 1997).

Binding May Be Associated with Unfolding. While partial structure appears to be present both in conformers recognized by GroEL and in stably bound substrate proteins, it has been unclear whether the act of binding promotes conformational change (i.e., partial unfolding), which could facilitate the removal of substrate polypeptides from kinetically trapped states. Support for this hypothesis comes from kinetic studies with MDH, which indicate that GroEL captures nonnative conformations and catalytically unscrambles these species through the act of binding them (Ranson et al., 1995). Furthermore, a protease-resistant 17 kDa domain of rhodanese, formed at the ribosome if release was prevented, became entirely protease susceptible upon release with puromycin and binding by GroEL, implying that unfolding had occurred in association with binding (Reid and Flynn, 1996). Likewise, deuterium exchange analysis revealed that barnase, a 6 kDa protein, became subject to transient global unfolding by addition of catalytic amounts of GroEL (Zahn et al., 1996). Such observations raise the possibility that GroEL can catalyze local unfolding of polypeptides by employing the energy of binding to lower the energy barrier for partial unfolding of a kinetically trapped state. Alternatively, GroEL may passively bind particular less-folded conformations of certain substrate proteins and, through the act of sequestering and ultimately allowing these to fold, shift the equilibrium of a mixture of nonnative, aggregation-prone species toward states that can fold (Walter et al., 1996). Both active and passive modes of binding may be operative in a physiological setting, where, for any given protein, the mode involved would be determined by the particular conformers that are present.

A further opportunity for unfolding after initial polypeptide binding has recently been suggested to exist during ATP–GroES binding, when a bound polypeptide could potentially be subject to stretching forces exerted by the twisting apical domain movements, prior to complete release into the cavity (see Figure 8 and Lorimer, 1997). The rapid drop of tryptophan fluorescence anisotropy in substrate protein in the first second after addition of GroES–ATP may reflect such unfolding (Rye et al., 1997). In energetic terms, formation of the stable GroEL–GroES–ATP complex may supply the energy for further unfolding of bound conformations.

Polypeptide Folding in the cis *Channel of GroEL—Infinite Dilution or Close Confinement?*

The nature of the path taken to the native state within the sequestered space of the *cis* cavity (Figure 7) remains a great unknown. It has been suggested that folding in this space occurs as if at infinite dilution, where there would be no interference from other molecules and no opportunity for multimeric aggregation (Agard, 1993). Insofar as the substrate protein is sequestered in a hydrophilic cavity, this may be so, but the walls of the cavity appear to interact physically with sequestered proteins, even after they have reached the native state (Weissman et al., 1996). Thus, the cavity walls act to confine the space, at least for larger substrates, and may limit the population of folding conformers to particular collapsed states. Defining such conformers and establishing whether they are the same as or different from those produced during folding in solution is an important, though technically challenging, task.

Whatever the particular conformers produced inside GroEL–GroES, it appears clear that this is a privileged environment. Even the most stubborn GroEL substrates, such as RUBISCO or MDH, of which only a few percent of molecules renature during any given normal lifetime of the complex, refold nearly completely in the *cis* space if the lifetime of the *cis* complex is prolonged, as in single-ring mutants (Rye et al., 1997). Thus, substrates in the active *cis* complex do not irreversibly misfold but, rather, ultimately find their way to the native state if given an extended period of time.

Rebinding of Nonnative Forms by Chaperonin

Under normal conditions, however, the folding-active complex has a lifetime limited to 15–20 s (perhaps less, in vivo). Presumably this time represents an evolved compromise between recovery in native form of only a fraction of "stubborn" substrate molecules and the need to rapidly release folded forms to carry out work in the cell. Those molecules that fail to reach native form at GroEL or in solution after release from it are either rebound by other GroEL molecules (or perhaps the same GroEL molecule, since it is most proximal) or by other chaperones (Weissman et al., 1994; Smith and Fisher, 1995; Taguchi and Yoshida, 1995; Buchberger et al., 1996; Burston et al., 1996; Todd et al., 1996; Ranson et al., 1997). In the case of rebinding to GroEL, it appears, provisionally, that rebound substrates occupy conformations similar to those originally bound. For example, rhodanese molecules released in nonnative form and captured by chaperonin "trap" mutants, able to bind but not release substrate, exhibited the same partial protease protection and tryptophan fluorescence as when originally bound (Weissman et al., 1994). Likewise, deuterium exchange/mass spectrometry studies of DHFR during GroEL-mediated refolding indicated no progression of protection in species that were rebound (Groβ et al., 1996). Thus, the individual reaction cycles appear to be all-or-none processes for at least these substrates.

Efficiency of the Chaperonin Machine and Constraints

The expenditure of 7-molecule packets of ATP (>50 kcal/mol each) by GroEL enables it to mobilize polypeptide substrates across as-yet-undefined energy landscapes, whose barriers are nonetheless probably no more than a few kcal/mol. Viewed in this way, the work appears expensive. Yet, the overall energy cost in folding a newly translated protein like RUBISCO, even assuming a need for multiple cycles of interaction, is no more than approximately a tenth of that for translating the same polypeptide chain. Thus, the evolutionary acquisition of a chaperonin folding machine, whose presence permitted the coevolution of a range of protein folds and functions that would otherwise not have been possible, at a cost of only 10% that of translation, seems like a good bargain.

Yet there are constraints. Bound proteins larger than 60–70 kDa probably prevent GroES binding in *cis* and thus cannot be assisted in a *cis* cavity the way smaller substrates are. One way around the size constraint would be to provide a co-chaperonin with a longer mobile loop and a taller dome, contributing to enlargement of the *cis* cavity size; recent structural studies indicate that the bacteriophage T4 has accomplished just this by encoding its own co-chaperonin (gp31) that uniquely assists productive folding of its large-sized capsid protein (gp23) (Hunt et al., 1997). It remains possible for other large(r) polypeptides, however, that binding in the *trans* ring might be associated with local unfolding of kinetically trapped regions that could allow proper folding to ensue upon release (see, however, Gordon et al., 1994).

In addition to size constraint, it is clear that some proteins simply cannot be assisted, for reasons as yet unknown. For example, GroEL efficiently binds vertebrate actin and, in the presence of GroES and nucleotide, the actin becomes enclosed in the *cis* channel, but it fails to achieve the native state. Rather, it nonproductively cycles from one GroEL molecule to the next (Tian et al., 1995). Even in single ring GroEL mutants, actin can be held indefinitely in the *cis* channel, but once GroES is released, it emerges in nonnative form (G. Farr, personal communication). By contrast, actin is efficiently folded by the chaperonin of the eukaryotic cytosol (Gao et al., 1992). Here there is no co-chaperonin involved, but instead, based on structural analysis of the apical domain of a related thermophilic archaebacterial chaperonin, there may be a built-in dome structure formed from protrusions emerging from each of the surrounding apical domains (Klumpp et al., 1997). Perhaps nonnative actin is bound by hydrophobic surfaces on the underside of this structure, producing a geometry of binding that is different from that of substrates bound and productively folded by GroEL. However, the converse case is even more dramatic: substrates of GroEL diluted from denaturant, such as rhodanese and MDH, are not recognized at all by the cytosolic chaperonin. There is thus at least some evidence for coevolution of chaperonins and their substrates. Whether this has reached any level of fine tuning of binding or adjustment of the timing of the reaction cycle is unclear. Presumably, any significant adjustments in favor of one substrate might compromise others, although this may be what occurred in the evolution of the eukaryotic cytosolic chaperonin, because its major substrates appear to be actin and tubulin with, it seems, only a few others.

Whither Mechanistic Study?

The foregoing mechanistic portraits of the two ubiquitous and essential chaperone systems, Hsp70 and Hsp60,

reveal the shared properties of recognition of hydrophobic surfaces in nonnative species and of ATP binding-directed substrate release, while at the same time articulating the different structural contexts of polypeptide binding by these chaperones, as well as the different mechanical actions of ATP binding and hydrolysis and influence of co-chaperones in driving the chaperones through their conformational states. At this point, further mechanistic investigation must include additional structural determinations that, in the case of Hsp70, define the interaction between ATPase and peptide binding domains in the various nucleotide states, as well as defining the nature of interaction of DnaJ both with substrate polypeptide and with Hsp70. In the case of the chaperonin system, a GroEL–GroES–ATP structure, defining activation of the folding-active state, seems desirable, as would be structures, even at EM-level resolution, visualizing substrates bound in the central channel. Beyond examining the chaperones themselves, the key structural work at this point really lies with the substrates. There is a need to define the conformations of substrate that are recognized, the conformational changes that occur attendant to stable binding, and the conformational changes that ensue with subsequent ATP-triggered substrate release. In energetic terms, one would like to be able to map resolvable nonnative conformations onto an energy surface and correlate the energetics with the events in the chaperone cycle. Likewise, the energetics of the chaperone states themselves would be desirable to resolve. New tools, both experimental and predictive, may be required.

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Note Added in Proof

Figure 6 is from Fenton et al., 1994, with permission; Figures 7 (adapted) and 8 are from Xu et al., 1997, with permission.