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**REVIEW**

# GroEL-mediated protein folding

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**Abstract**



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The early studies of Anfinsen and co-workers established that the primary structure of a protein contains all the information necessary to direct the native secondary and tertiary fold (Anfinsen, 1973). Under physiologic conditions inside the cell, however, the folding process for many proteins, particularly those with multidomain structures, is prone to producing a variety of misfolded species and aggregates. Recent studies indicate that a specialized class of proteins, known as molecular chaperones, plays an essential role in the cell, binding non-native proteins and preventing protein aggregation. The large double-ring structures of the subclass known as chaperonins have been of particular interest be-

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cause, in addition to binding non-native states, they also facilitate ATP-dependent folding to native form of a large number of newly translated and newly translocated polypeptides. Two families of chaperonins have been identified: the GroEL/Hsp60 family, found in the cytoplasm of eubacteria and the soluble matrix of mitochondria and chloroplasts; and the TF55/CCT family, found in the cytoplasm of archaebacteria and the cytosol of eukaryotes. GroEL from *Escherichia coli* is the most thoroughly studied chaperonin, and its mechanism provides the paradigm for chaperonin-assisted protein folding.

Recent structural and functional studies of GroEL suggest that its role in the cell is to provide an environment that facilitates proper folding, namely its central channel. In particular, GroEL seems to perform two actions in assisting folding, each associated with a specific conformational state of chaperonin. One is the *binding* of collapsed, partially structured folding intermediates in the GroEL central channel, forestalling aggregation. Such binding can be associated with structural rearrangement amounting to partial unfolding of non-native forms recognized through their exposure of hydrophobic surface. The other action is *facilitation of folding*, occurring inside the central channel after enclosure by the cochaperone, GroES, in the presence of ATP. During the lifetime of this folding-active ternary complex, polypeptides initiate folding, and a fraction reaches either native form or a conformation committed to achieving the native state. The remaining fraction of polypeptide in such ternary complexes fails to reach a committed state, however. Following ATP hydrolysis and discharge of GroES, these non-native polypeptides can release, along with native and committed forms, into the bulk solution, where they partition kinetically among chaperones and proteolytic components. Such partitioning, which can include rebinding to GroEL, determines the fate of non-native proteins within a cellular compartment. We discuss below the structural and functional studies that have led to the current view and identify aspects of chaperonin mechanics that are not, as yet, understood.

## I. Architecture of GroEL and GroES and the reaction pathway

### *A. Architecture of the chaperonins*

GroEL is a large homotetradecamer composed of two back-to-back seven-membered rings of 57-kDa subunits (Fig. 1A). The crystal structure of unliganded GroEL refined at 2.8 Å from an orthorhombic crystal form shows a cylindrical complex 145 Å in height and 135 Å in diameter, with a central channel or cavity at either terminus measuring 45 Å in diameter (Braig et al., 1994, 1995). Electron microscopy studies have shown that non-native polypeptides are bound in these channel openings (Langer et al., 1992; Braig et al., 1993; Chen et al., 1994; Ishii et al., 1994). Because the amino acids forming the walls of the channel are hydrophobic in character (Fig. 1B), and because their substitution with polar amino acids abolishes polypeptide binding (Braig et al., 1994; Fenton et al., 1994), it appears that hydrophobic contacts between the channel wall and the exposed hydrophobic surface of non-native folding intermediates mediate polypeptide binding (see below for further discussion).

The GroEL subunit is composed of 547 amino acids folded into three domains. Collectively, the *apical* domains ("A" in the right panel of Fig. 1A) form the end portion of the chaperonin cylinder, with the channel-facing aspect involved with polypeptide and GroES binding (Fig. 1B). The domain is composed of an orthogonal $\beta$-sheet structure flanked at its inside and outside by $\alpha$-helices. This is the least-resolved of the three GroEL domains in two different crystal forms of GroEL, suggesting an intrinsic flexibility that appears to be functionally necessary to accommodate binding of a large variety of different polypeptides in the central channel. Such flexibility could occur at two levels. On one hand, the entire apical domain of a given subunit might move as a rigid body, so that the collective of apical domains around the ring could accommodate asymmetry of bound substrate proteins. At another level, flexibility could occur locally within the apical domain, for example, in the channel face, to accommodate to the conformation of a bound polypeptide.

The collective of *equatorial* domains (marked "E") forms the structural foundation of the cylinder, providing the major contacts between subunits both within and between rings. The domain is composed of long, nearly parallel $\alpha$-helices. The peptide chain resolvable in the crystal structures terminates at residue 523, at the channel-facing aspect of this domain, leaving the 24 COOH-terminal residues of the protein unresolved. They are apparently disordered, as might be predicted by the occurrence of a GGM repeat through the last 15 amino acids. Electron microscopy has indicated that there is density within the central channel at the equatorial level in both rings, likely accounting for these residues. Furthermore, small-angle neutron scattering studies show that the COOH-terminal residues in aggregate close the central cavity at the equator (Thiyagarajan et al., 1996). The ATP binding site of each subunit lies in the top surface of the equatorial domain at the channel-facing aspect (Fig. 1C). The nucleotide site is comprised of a set of highly conserved loops, including the sequence GDGTT, conserved in all chaperonins. Interactions of nucleotide bound in this site have been analyzed in a monoclinic crystal of ATP$\gamma$S complexed with GroEL, determined at 2.4 Å (Fig. 1D). This shows that the GDGTT sequence is a phosphate-binding loop interacting both directly and indirectly, via a bound $Mg^{2+}$, with oxygens of all three phosphates (Boisvert et al., 1996).

As yet, there is no clear understanding at the structural level of the mechanism of ATP hydrolysis or the allosteric consequences of ATP binding/hydrolysis. Speculations can be raised, however, about the routes of allosteric transmission of signals from the ATP site to the subunits of the opposite ring. There appear to be two major sites of contact of each GroEL subunit with adjacent subunits across the equatorial plane in the opposite ring: one between K105, A109, and E434 of one subunit with A109 and E434 of a subunit in the opposite ring (so-called left site with reference to a subunit of the top ring viewed from outside), and the other between E461, S463, and V464 with R452, S463, and V464 of the next door neighbor in the opposite ring (so-called right site). Interestingly, the phosphate binding loop (87–91) is immediately adjacent in the primary sequence to the $\alpha$-helix and adjoining loop that bear K105 and A109, which participate in the first contact. Recent electron microscopy studies have suggested that, in the presence of ATP, this contact becomes less resolvable, indicating that it may indeed be a line of movement linking ATP binding with distant conformational changes (Roseman et al., 1996).

The *intermediate* domain (marked "I") is a smaller domain forming a hinge-like covalent connection between the apical and equatorial domains. Inspection of GroEL in different conformational states both by electron microscopy (Roseman et al., 1996) and by comparison of X-ray structures of unliganded GroEL and a GroEL–GroES complex support the hypothesis that hinge-like motions

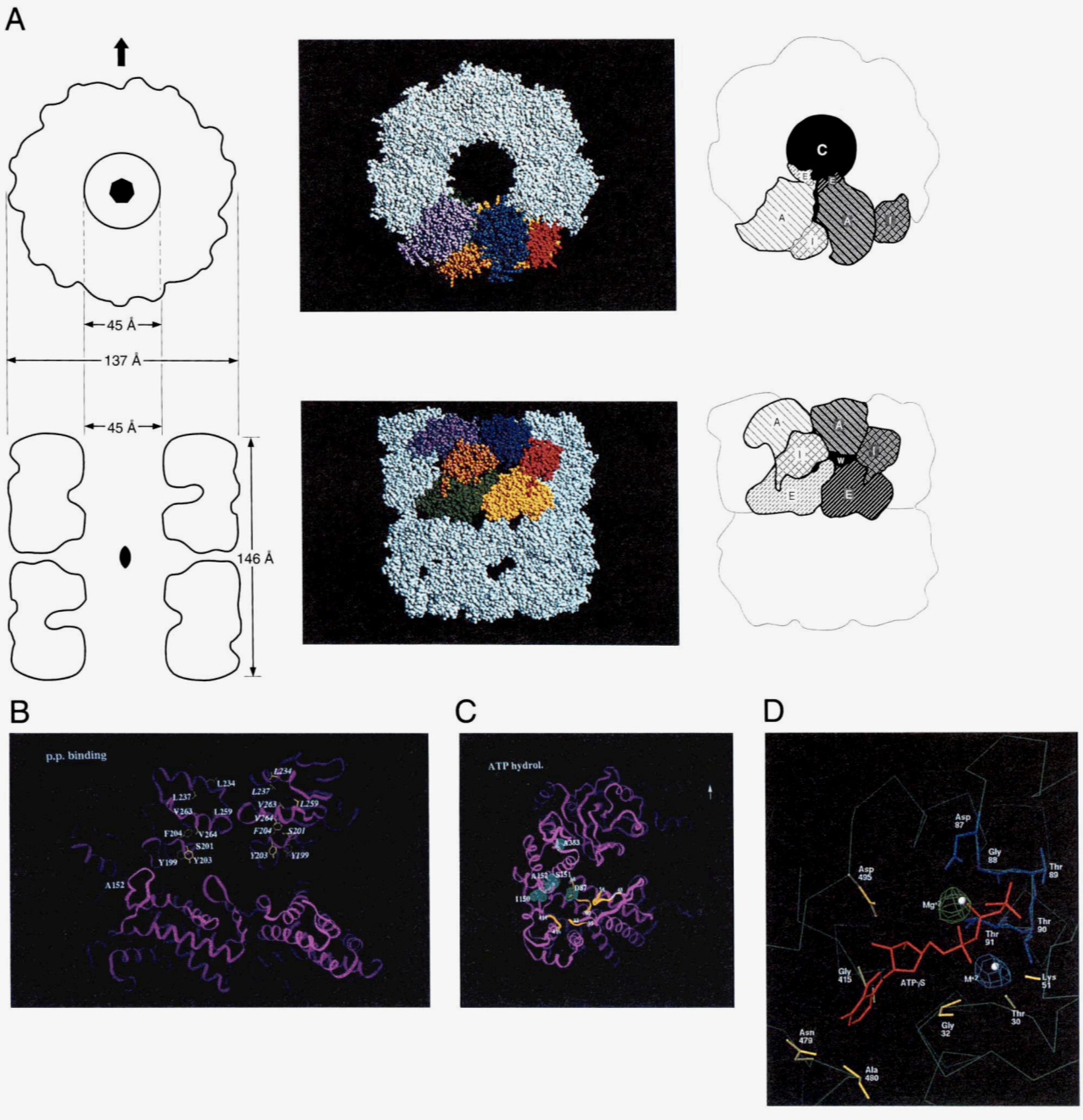


**Fig. 1.** GroEL architecture. **A:** The overall structure of the GroEL tetradecamer is shown in two views: from the top, looking into the central cavity; and from the side. The left panel gives the dimensions of the cylinder. The middle panel is a van der Waals space-filling model of the chaperonin, with two of the subunits in the upper ring colored to show the three domains, apical, intermediate, and equatorial: left subunit, purple, gold, green; right subunit, blue, red, yellow, respectively. The right panel is a diagrammatic representation of the middle one, with the domains designated A (apical), I (intermediate), and E (equatorial); also identified here are the central cavity (C) and one of the side openings (W). From Braig et al. (1994), with permission. **B:** Residues in the channel face implicated in polypeptide and GroES binding (side chains in yellow) are shown for two GroEL subunits, superimposed on ribbon diagrams of the backbone $\alpha$-carbon trace (purple). The view is from inside the central channel of the top ring in A; one subunit's residues are denoted in italics. From Fenton et al. (1994), with permission. **C:** ATP binding site and residues affecting ATP hydrolysis (green van der Waals spheres) are shown in a side view of a single subunit (purple backbone ribbon) from the upper ring; the central channel is to the right, outside is to the left. Residues around the binding pocket that are conserved are shown with small numbers and yellow coloring of the backbone. From Fenton et al. (1994), with permission. **D:** High-resolution view of the ATP binding site is shown, with ATP$\gamma$S (red) as the ligand. The backbone $\alpha$-carbon trace is cyan, residues involved in the phosphate binding loop are shown with side chains in dark blue, and other residues interacting with the nucleotide or the second cation are in yellow. The two cations are shown as white spheres, with the anomalous difference contour for $Mg^{2+}$ shown in green and the electron density contour for the second cation ($M^{2+}$) shown in light blue. Figure courtesy of D. Boisvert.

center on this domain, suggesting that this is a major line of allosteric communication. An additional route is afforded by a noncovalent connection formed between the intermediate domain and the apical domain of the neighboring subunit to the right (considering the top ring viewed from outside; see Fig. 1A) that appears to be critical to the observed positive cooperativity of nucleotide binding and hydrolysis within GroEL rings and negative cooperativity between rings (Yifrach & Horovitz, 1994). Mutations within the intermediate domain can affect any and all of the measurable functions of GroEL, consistent with a major role in signaling (Fenton et al., 1994).

GroEL's cochaperonin partner, GroES, is a smaller structure, a single seven-membered ring of 10-kDa subunits (Hunt et al., 1996; Mande et al., 1996) (Fig. 2) that binds at one or both ends of the GroEL cylinder in the presence of nucleotide (Chandrasekhar et al., 1986; Saibil et al., 1991; Langer et al., 1992; Schmidt et al., 1994b), producing an upward and outward opening of the GroEL apical domains, which nearly doubles the volume of the central channel (Fig. 3) (Chen et al., 1994). Crystal structure analyses of GroES reveal a dome-shaped structure about 70–80 Å in diameter and 30 Å high, with inside dimensions of ~30 Å diameter and ~20 Å high. Each GroES subunit has a core $\beta$-barrel structure (Fig. 2, bottom) giving off two $\beta$-hairpin loops, one arching upward and inward from the top aspect, to contribute to the top of the dome, and a second, at the lower lateral aspect, that interacts with a GroEL subunit, probably on a 1:1 basis with residues in the apical channel face. Given the atomic features of GroES and electron microscopy images of GroEL–GroES binary complexes, it is evident that, in GroEL–GroES complexes, the interior cavity of GroES becomes continuous with that of the GroEL central channel (Fig. 3). The contacting loops of GroES are nonordered and flexible in unbound GroES, as indicated by elegant studies mapping a sharp NMR signal to this region of free GroES in solution (Landry et al., 1993); the same loop region becomes ordered upon binding to GroEL, as revealed by broadening of the NMR signal. Correspondingly, this mobile loop region fails to resolve in the crystal structures of unbound GroES, with the exception of one loop of the seven held in a rigid position by a crystal packing contact (Hunt et al., 1996).

The suggestion that the GroES mobile loops at least in part bind to and compete for the same surface of GroEL as polypeptide is supported both by observations in electron microscopy of the site of contact (Chen et al., 1994; Roseman et al., 1996) and by mutational findings that the same channel-facing residues critical for polypeptide binding (Fig. 1B) are also critical for binding GroES (Fenton et al., 1994). Such apparent competition by GroES for the peptide binding sites leads, in the presence of ATP, to complete release of polypeptide into the central channel, accompanied by initiation of productive folding (Weissman et al., 1995, 1996; see below).

### *B. Reaction pathway of GroEL–GroES-mediated folding*

Our current understanding of the pathway of a chaperonin reaction is summarized in Figure 4. (1) Non-native polypeptide ($I_{uc}$) is bound by an asymmetric GroEL–GroES complex, a dynamic species formed in the presence of physiological levels of ATP/ADP, in which the GroEL central channel at one end of the cylinder is capped by GroES, whereas the channel at the other end remains accessible to polypeptide (Jackson et al., 1993; Martin et al., 1993; Burston et al., 1995; Weissman et al., 1995; and see below).

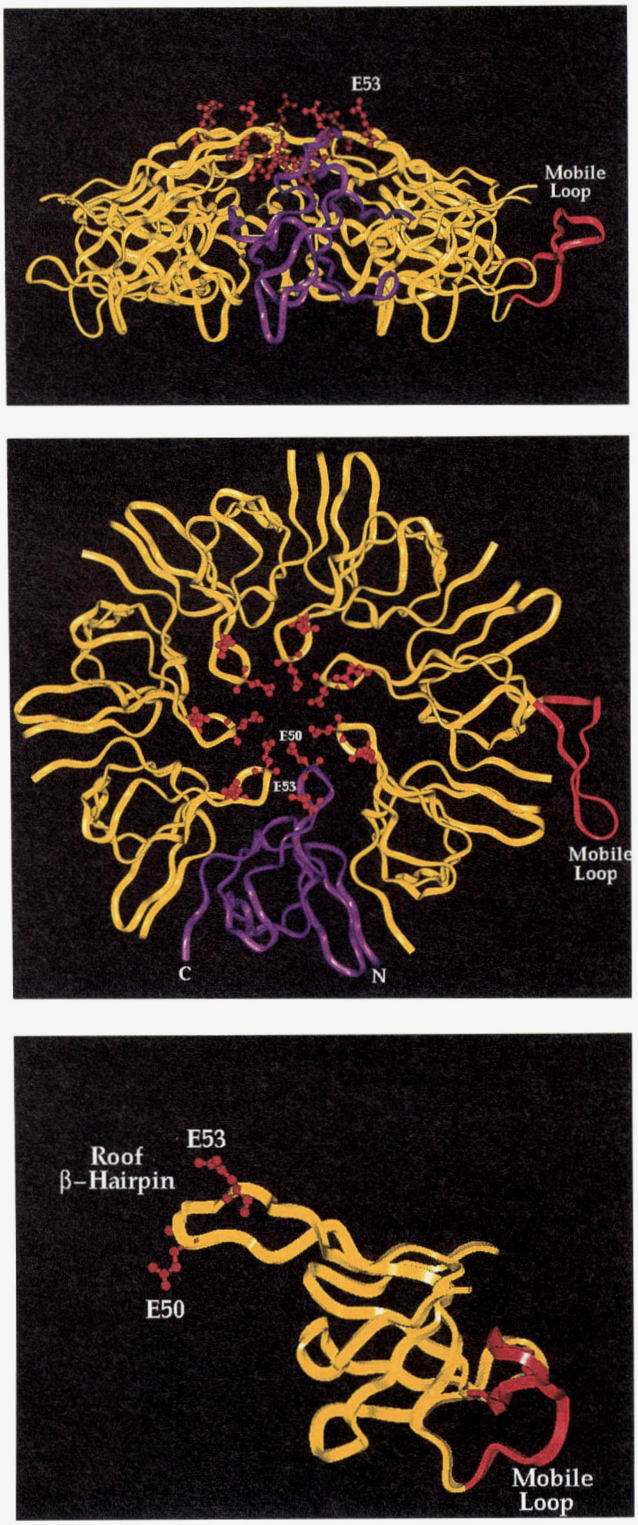


**Fig. 2.** GroES architecture. Side (upper panel) and top (center panel) orthogonal views of the GroES heptamer as a ribbon diagram (yellow). One subunit is shown in purple, and the single resolved mobile loop is shown in red. Also in red are ball-and-stick representations of the roof $\beta$-hairpin glutamates, E50 and E53. The amino (N) and carboxyl (C) termini of the purple subunit are indicated. In the lower panel, the monomer of GroES with the resolved mobile loop is shown, colored similarly. This view is rotated about 45° relative to that of the same subunit in the upper panel to show the central barrel and roof $\beta$-hairpin clearly. From Fenton et al. (1996), with permission.

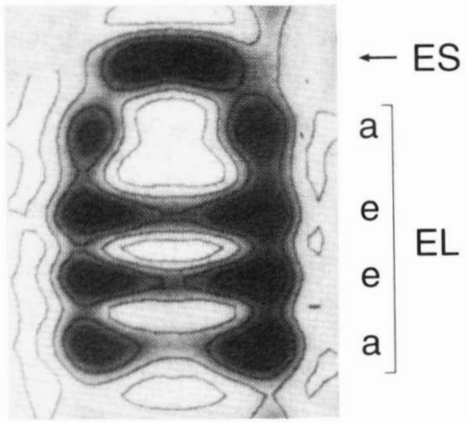


**Fig. 3.** GroEL–GroES binary complex. A section through the three-dimensional map of the asymmetric GroEL–GroES complex, generated by cryo-electron microscopy and image averaging, is shown. Apical (a) and equatorial (e) domains of GroEL (EL) are indicated. Note the difference in volume between the upper channel space under GroES (ES) and the lower unliganded channel. Redrawn from Chen et al. (1994), with permission.

(2) GroES becomes bound to the same ring as polypeptide, forming a productive *cis* ternary complex (Bochkareva & Girshovich, 1992; Weissman et al., 1995; Mayhew et al., 1996). Such a *cis* ternary complex could be formed either by release of GroES from the *trans* ring, followed by binding in *cis*, or by binding of a second GroES in *cis*, forming a "football" structure. Although such symmetric complexes have been observed (Azem et al., 1994; Schmidt et al., 1994b), their participation does not seem to be obligatory in vitro (Hayer-Hartl et al., 1995; Weissman et al., 1995; see below). On the other hand, their formation may be favored in the cell. Importantly, the binding of GroES to GroEL leads to a doubling in volume of the central channel in the bound GroEL ring, as the result of opening of the GroEL apical domains to contact GroES (Fig. 3; Chen et al., 1994; Roseman et al., 1996). Thus, non-native polypeptides of up to ~60 kDa can be accommodated in this central space underneath GroES. (3) In the presence of ATP, polypeptide bound in the *cis* ternary complex is released from its binding sites, initiating folding inside the sequestered space underneath GroES (Weissman et al., 1996). (4) Quantized hydrolysis of seven ATPs in the equatorial domains of the subunits of the *trans* ring, opposite GroES and polypeptide, leads to discharge of GroES after ~15–30 s (Todd et al., 1994; Burston et al., 1995; Hayer-Hartl et al., 1995), giving polypeptide the chance to depart in any of three different conformational states: the native state ($N$), a conformation committed to reaching the native state in solution ($I_c$), or an uncommitted, non-native form ($I_{uc}$). The non-native polypeptides ($I_{uc}$) can then rebind to GroEL for another attempt at folding. A more detailed treatment of each aspect of this pathway follows.

## II. Polypeptide binding

### *A. Parallel network of chaperones binding polypeptides in vivo*

In the cell, chaperones have been observed to interact with newly translated polypeptides both during translation and after release from ribosomes. For example, in the eukaryotic cytosol, both genetic and biochemical evidence support interaction of Hsp70 class proteins with nascent polypeptide chains (Beckmann et al., 1990; Nelson et al., 1992; Frydman et al., 1994). Hsp70s interact with substrate proteins by binding short extended segments of polypeptide that are rich in hydrophobic amino acids (Flynn et al., 1989, 1991; Blond-Elguindi et al., 1993; Gragerov & Gottesman, 1994; Gragerov et al., 1994; Knarr et al., 1995; McCarty et al., 1996). Yet, in the bacterial cytoplasm, no cotranslational interaction has been observed either with the Hsp70 homologue, DnaK, or with

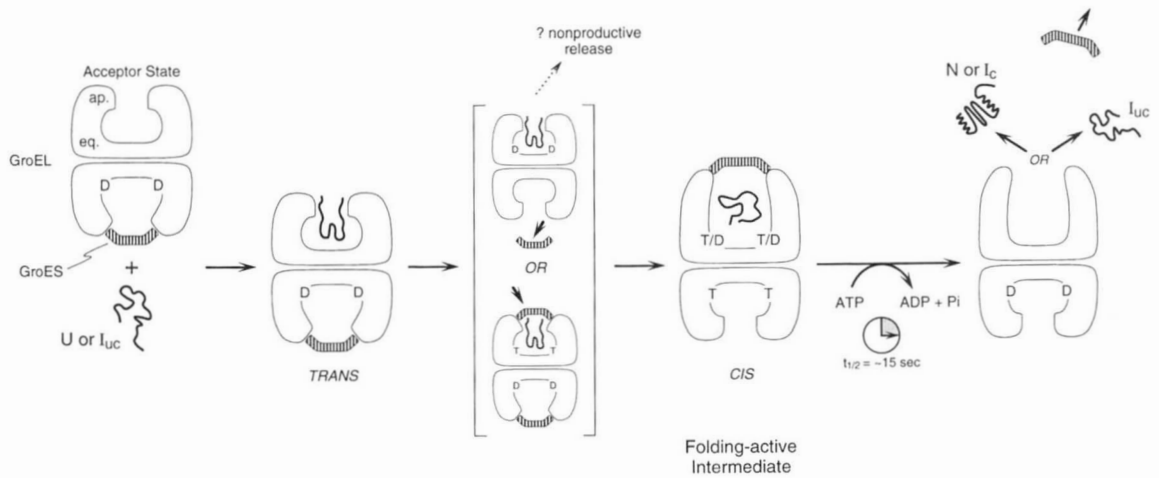


**Fig. 4.** Model for a GroEL–GroES-mediated folding reaction. The asymmetric GroEL–GroES complex (first panel: ap., apical domain; eq., equatorial domain) is the likely polypeptide acceptor state in vivo, and binds unfolded polypeptides ($U$) or kinetically trapped folding intermediates ($I_{uc}$) to form the *trans* ternary complex (second panel). This complex is highly dynamic with respect to GroES binding in the presence of ATP; two of the possible pathways of GroES release and rebinding that lead to the *cis* complex are shown (third panel). When polypeptide is sequestered underneath GroES in the presence of ATP (the folding-active *cis* intermediate, fourth panel), major conformational changes occur in the *cis* GroEL ring, and polypeptide folding is initiated. Simultaneously, ATP binding in the *trans* ring starts the timer for hydrolysis and release. When ATP hydrolysis occurs in the *trans* ring ($t_{1/2}$ ~ 15 s), GroES is released, giving polypeptide the opportunity to depart (last panel). 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 and T, ATP. Redrawn from Fenton et al. (1996), with permission.

GroEL (e.g., Reid & Flynn, 1996). Rather, a major cotranslational interaction has been identified recently with a component known as trigger factor, which has peptidyl prolyl isomerase activity (Stoller et al., 1995; Valent et al., 1995; Hesterkamp et al., 1996).

GroEL thus appears to function posttranslationally, binding to polypeptide chains that have been released from the translation machinery or from other components, perhaps including Hsp70 proteins. Although there has been considerable argument advanced for a sequential pathway of interactions proceeding from Hsp70s to Hsp60s (Hartl, 1996), proteins reaching GroEL in the bacterial cytoplasm do not have such a requirement because, for example, *E. coli* cells are viable in the complete absence of DnaK (Hsp70), as long as they are maintained below 30 °C (Paek & Walker, 1987; Bukau & Walker, 1989). This means that, under these conditions, there is not a single essential protein that requires DnaK action to reach its native state. Conversely, it can also be concluded that those proteins that critically depend on GroEL for productive folding at all temperatures, including 30 °C, have no absolute requirement for an initial interaction with DnaK. Thus, the cell apparently does not require a sequential pathway of interactions.

In further support of parallel pathways of chaperone action are studies of mutant *E. coli* strains in which the heat shock transcriptional regulator, $\sigma 32$, is defective (Gragerov et al., 1992). This results in deficiency of all the heat shock proteins, including DnaK and GroEL, and leads to wholesale protein aggregation. Importantly, overexpression of either the GroEL/GroES or the DnaK/DnaJ pair (from a *lac* promoter) in this setting forestalls aggregation of the same large collective of proteins. Thus, either component may be able to act on a large class of proteins exclusive of the other, at least in a setting of overproduction.

Notably, in contrast with survival of cells deleted of DnaK, deletion of GroEL is lethal at all temperatures (Fayet et al., 1989). This suggests that at least one essential protein requires the action of GroEL at all temperatures. A broader range of substrates may be involved, however, as suggested by studies both in vitro, where ~40% of the protein species in a radiolabeled denatured *E. coli* extract were found to associate with GroEL after dilution (Viitanen et al., 1992), and in vivo with a temperature-sensitive mutant of GroEL, in which as many as 30% of newly translated bacterial proteins failed to reach native form (Horwich et al., 1993) (for a theoretical assessment of this problem, see Lorimer, 1996).

In addition to the posttranslational interactions with GroEL that occur under normal conditions, polypeptides also interact with GroEL under conditions of thermal or chemical stress. Under these conditions, native proteins are subject to unfolding, and the resulting aggregation-prone intermediates can be bound by GroEL and may ultimately, upon return to normal conditions, be refolded to native form or released to other chaperones or the proteolytic machinery (e.g., Höll-Neugebauer et al., 1991; Martin et al., 1992; Mendoza et al., 1992b; Kandror et al., 1994). Whether GroEL originally evolved to provide the function of thermal protection is unclear. Alternatively, it might have arisen in the context of folding at normal temperature, permitting the efficient folding and, hence, evolutionary success, of new protein conformations that conferred unique catalytic or structural functions. Whichever came first, the net results, in terms of contemporary function, were twofold: on the one hand, an ability to provide for protein and cellular survival under stress, and on the other, the opportunity for a cell to routinely use a broader region of "conformational space" for deriving catalytic and structural functions than would be available if folding were only spontaneous.

### *B. Polypeptide binding in vitro*

It seems likely that refolding studies conducted in vitro, typically involving dilution of unfolded, full-length proteins from denaturant, can produce the same spectrum of intermediates that is produced when fully translated proteins are released from the bacterial ribosome, because GroEL does not cotranslationally bind to proteins. Indeed, binding to GroEL in vitro has recapitulated the prevention of aggregation observed in vivo. For example, binding by GroEL of *Rhodospirillum rubrum* ribulose bisphosphate carboxylase (RUBISCO), diluted from denaturant, competed with aggregation of the refolding protein (Goloubinoff et al., 1989). In the case of the mitochondrial protein, rhodanese, aggregation was prevented not only by GroEL, but also by the detergent, lauryl maltoside, raising the possibility that the action of GroEL in binding intermediate forms was to stabilize exposed hydrophobic surfaces (Mendoza et al., 1991). In vitro studies can thus be informative concerning GroEL binding and folding functions. What are the characteristics of non-native polypeptides that are recognized by the chaperonin? Is overall hydrophobicity the sole feature recognized? Do secondary or tertiary structural elements play a role as well? Are there individual amino acids or specific motifs that are preferred?

#### *1. Role of hydrophobicity in recognition*

Because one of the distinguishing features of folding intermediates, such as molten globules, is the increased exposure of hydrophobic regions to solvent, it seemed likely that hydrophobicity might be important for chaperonin recognition. The structure and functional studies of GroEL support this idea. For example, the apical channel face is formed by a tier of two $\alpha$-helices and an underlying extended loop segment from each subunit, largely composed of hydrophobic amino acids, generating a hydrophobic surface almost 30-Å high around the entire channel (see Fig. 1B). Moreover, the residues in these elements were poorly determined and appear highly mobile in the X-ray structures, with large *B*-factors, suggesting an ability to move to accommodate a variety of individual polypeptide ligands (Braig et al., 1994, 1995; Boisvert et al., 1996). Finally, mutations changing hydrophobic residues in these regions to charged or hydrophilic ones resulted in mutant GroELs that were unable to bind polypeptide substrates (Fenton et al., 1994).

In regard to the substrates, a number of studies have examined the contributions of hydrophobicity, using a spectrum of molecules. Richarme and coworkers observed that hydrophobic amino acids in solution could increase the ATPase rate of GroEL two- to threefold at concentrations of about 0.5 mM and that this effect was reversed by the presence of GroES (Richarme & Kohiyama, 1994; de Crouy-Chanel et al., 1995). Landry and Gierasch (1991) synthesized a 13-residue peptide, comprising the $NH_2$-terminus of rhodanese, which, although not particularly hydrophobic, appeared to bind to GroEL, as monitored by NMR spectroscopy. Interestingly, although the peptide was largely unstructured in solution, it appeared to form an $\alpha$-helix when bound to GroEL. Another short peptide, vsv-C, perhaps somewhat more hydrophobic, also binds to GroEL and forms an $\alpha$-helix, although, in this case, the solution structure of the peptide is already somewhat helical (Landry et al., 1992). Two considerably larger peptides have been generated by mild protease digestion of rhodanese while bound to GroEL, on the assumption that bound segments would be more protected from proteolysis. The stable peptides recovered (~7 kDa and 11 kDa) corresponded to two $\alpha$-helical regions in native rhodanese that

exhibit hydrophobic faces that ultimately interact with each other in the native form, and these were speculated to be the sites of binding to GroEL (Hlodan et al., 1995). Finally, a mutagenesis study examined the effects of various substitutions on binding of the small protein, chymotrypsin inhibitor 2 (CI2) (Itzhaki et al., 1995). Retardation of folding in the presence of GroEL was taken as a measure of binding. There was a general trend toward less binding with replacement of hydrophobic residues by alanine or glycine, but the effects were small (<10-fold); likewise, there was a correlation between the retardation of the folding rate constant and the change in the hydrophobicity of the side chain produced by the mutation.

A different approach to assessment of hydrophobic interaction was reported by Lin et al. (1995), who used isothermal titration calorimetry. A stably unfolded variant of subtilisin, BPN′ PJ9, bound to GroEL with a positive enthalpy change (+19.9 kcal/mol), implying that binding was entropy-driven, and a negative heat capacity change, $\Delta C_p$ ($-0.85$ kcal mol$^{-1}$ deg$^{-1}$), diagnostic of hydrophobic interaction. The naturally disordered protein, $\alpha$-casein, showed a similarly negative $\Delta C_p$, suggesting that its binding also was driven by hydrophobic interactions. The authors calculated from the $\Delta C_p$ that approximately 3,000 Å$^2$ of nonpolar surface would be buried when BPN′ PJ9 bound to GroEL; interestingly, the hydrophobic surface of the apical channel face of GroEL is approximately 4,500 Å$^2$.

### 2. *Homologous proteins with differing recognition: Differences in primary structure versus effects on folding pathway*

Additional information on the possible sequence specificity of GroEL binding has come from examination of chaperonin-mediated folding of homologous proteins derived from different species or different cellular compartments. For example, whereas human DHFR is quantitatively recognized and bound by GroEL during its refolding in vitro, the *E. coli* protein, which has a virtually identical fold but only ~30% identity of amino acid sequence, is not recognized at all (Clark et al., 1996). The refolding human protein is highly aggregation-prone in the absence of GroEL, whereas the refolding *E. coli* protein is remarkably soluble, even at high concentrations (M.S. Goldberg & A.L. Horwich, unpubl. obs.). The study of Clark et al. focused on the observation that there are several loops in the structure of the *E. coli* enzyme that appear to be shorter than in the mammalian homologue. This suggested that these loops might be specific sites of recognition. To test this, loops corresponding to the mouse sequence were placed individually into two corresponding sites in the *E. coli* protein. The derived products were now recognizable by GroEL from either denatured or native states. The interpretation of these results is not unequivocal, however. Although the inserted loops could conceivably provide specific sites of recognition by GroEL, the insertions might more likely alter the kinetics of folding, populating different intermediate states on the folding pathway that expose hydrophobic surfaces that can be recognized by GroEL. Although they did not perform kinetic studies, Clark et al. noted that the native state of the derived proteins was destabilized, resembling the behavior of wild-type human DHFR, which gradually becomes bound by GroEL in the absence of ligand (Viitanen et al., 1991). Supporting the possibility that DHFR recognition involves more than simple provision of a direct binding site, a converse experiment has been carried out recently, in which the larger loops of human DHFR were trimmed individually or collectively to the size of those of the *E. coli* protein. All of the derived proteins bound as efficiently to GroEL as wild-type human DHFR, suggesting that the population of folding intermediates recognized is unrelated to the loops (M.S. Goldberg, unpubl.).

Effects of primary structural differences on affinity for GroEL have also been observed for pairs of cytosolic and mitochondrial isozymes, which have significant amino acid homology (~50%) and essentially identical three-dimensional structures: for example, aspartate aminotransferase (AAT) (Mattingly et al., 1995) and malate dehydrogenase (MDH) (Staniforth et al., 1994b). Mitochondrial AAT seemed to bind more tightly than the cytosolic isozyme when both were diluted from denaturant, even though the cytosolic enzyme has slightly more hydrophobic residues in its primary sequence. Likewise, mitochondrial MDH (mMDH) bound better than cytosolic, although in this case, the mitochondrial homologue has a considerably higher calculated hydrophobicity. Moreover, the GroEL–mMDH complex was more stable, requiring both GroES and ATP for efficient dissociation, whereas the complex with cytosolic MDH required only ATP. In an example from a mutational study, a set of amino acid substitutions were placed into a single position in yeast citrate synthase (CS) (Zahn et al., 1996a), and binding to GroEL was examined by competition with $\beta$-lactamase. Here, little difference in apparent binding constants was observed for the series A, P, L, K, D, all substituted for a particular glycine in CS. More generally, point mutations in many proteins appear to be able to elicit more efficient binding, relative to the wild-type protein, by GroEL (for example, Van Dyk et al., 1989; Gordon et al., 1994). The breadth of these effects and the lack of recognizable specificity in the affected residues argues more for an effect on the overall pathway or kinetics of folding, rather than for alteration of specific binding sites for GroEL. Thus, general hydrophobicity appears to be more important than specific residues as a determinant of polypeptide binding by GroEL.

### 3. *Conformations recognized by GroEL*

Although specific residues or binding sites do not not seem likely to be involved in GroEL recognition, the structural context of hydrophobicity could be important in determining which polypeptides bind to GroEL. For some polypeptides, it seems clear that any of several intermediate states can be recognized and bound by GroEL, albeit with different rates of association and affinities. The extent of hydrophobic exposure may determine the relative stability of these different complexes. In general, it seems that, for many proteins, the conformations that are preferred in bindng are collapsed but loosely packed, containing native-like secondary structure and, at least in some cases, a global topology that may be native-like; that is, an unstable form of the native fold may be present, lacking both some portions of secondary structure and a majority of stabilizing tertiary contacts. Such conformations generally expose more hydrophobic surface than the native state. On the other hand, kinetic, rather than thermodynamic, considerations may dictate where on the folding pathway GroEL binding occurs when a complete folding reaction occurs in vitro or in vivo.

*a. Refolding studies.* One approach to identifying what structures or conformations GroEL recognizes has been to allow refolding up to the point of stable binding by GroEL, which slows or halts further folding. As an alternative, polypeptides can be increasingly unfolded (by increasing temperature, for example) until GroEL binding occurs. In both cases, the intermediate has then been defined by physical or kinetic measures. For a variety of

substrate proteins diluted from denaturant, it appears that early, collapsed structures can be bound to GroEL. For example, when diluted from urea, pre-β-lactamase bound to GroEL and folding halted (Zahn & Pluckthun, 1992). Two kinetic intermediates were apparent in the refolding curves, perhaps representing a *cis–trans* proline isomerization, but both interacted with GroEL. Because these intermediates folded with the same rate and pH dependence when released from GroEL as in its absence, it was concluded that the same folding pathway was followed with or without chaperonin. The intermediate(s) had a fluorescence spectrum similar to native and quite different from that of the urea-denatured starting material. Thus, it seemed likely that a collapsed, native-like structure had been formed rapidly and was recognized. Interestingly, mature β-lactamase, lacking the signal peptide, did not bind to GroEL when diluted from urea, even when the folding buffer contained sufficient denaturant to populate a known folding intermediate (Zahn & Pluckthun, 1992; Zahn et al., 1994a). At elevated temperatures (>37 °C), however, mature β-lactamase unfolded into a form that bound tightly to GroEL (Zahn & Pluckthun, 1994). The fluorescence spectrum of this state was similar to that of GroEL-bound pre-β-lactamase. A recent, more-detailed examination of this bound intermediate, both by denaturation (monitored by Trp fluorescence) and by hydrogen-deuterium exchange (monitored by mass spectrometry), revealed considerable structure to be present, greater than that in the acid-induced molten globule conformation (Gervasoni et al., 1996). Furthermore, the observation that the apparent dissociation constant for the GroEL-β-lactamase complex decreased with increasing temperature indicated that hydrophobic interactions between β-lactamase and chaperonin were largely responsible for the binding (Zahn & Pluckthun, 1994). In another example, studies of refolding of a thermophilic lactate dehydrogenase (LDH) showed that different intermediate states could be populated upon dilution from various concentrations of guanidine hydrochloride. Only the refolding protein from guanidine concentrations producing either complete denaturation or an early intermediate form, a collapsed, molten globule-like state, interacted with GroEL (Badcoe et al., 1991). By contrast, neither a later intermediate, with more secondary structure, nor the folded monomer was bound by GroEL.

In our own studies of human dihydrofolate reductase (DHFR) refolding from denaturant (Goldberg et al., 1997), we observed by stopped-flow in the absence of GroEL a characteristic rise in DHFR tryptophan fluorescence in the first 100 ms, comprising two phases. One, the burst phase (<5 ms), was associated with acquisition of anilino-naphthalene sulfonate (ANS) binding competence, presumably the result of rapid collapse of the structure with exposure of nonpolar surfaces. The succeeding phase, associated with the rising tryptophan fluorescence, was apparently related to burial of one or more tryptophan side chains, likely reflecting formation of secondary or tertiary contacts. A subsequent slower decrease in fluorescence intensity, occurring over several minutes, correlated with production of the native state. This change was best described kinetically as reflecting two parallel folding pathways from independent intermediate states. When GroEL was present at 1 μM (with 1 μM DHFR), only the two late intermediates were bound, as indicated by a halt in the fluorescence change. At higher GroEL concentration (>10 μM), however, even the burst phase intermediate was bound, suggesting that competition between binding and folding was determining which intermediate was observed to interact with GroEL. Thus, the earliest form recognized and bound by GroEL was a collapsed, partially structured intermediate, but later intermediates, with more fully formed secondary structure, also were bound rapidly and stably.

*b. Binding of metastable intermediates.* An independent approach to analyzing what structures GroEL recognizes is to test the ability of preformed, metastable folding intermediates to bind to GroEL. Perhaps most revealing have been studies of α-lactalbumin. Initial work indicated that the relatively compact molten globule intermediate in the α-lactalbumin folding pathway, perhaps analogous to the later LDH intermediate mentioned above, was not bound by GroEL (Hayer-Hartl et al., 1994; Okazaki et al., 1994), but more recent experiments have shown that it does bind, albeit with a much-reduced association constant (Katsumata et al., 1996). Other, more expanded forms of α-lactalbumin, either the reduced state or scrambled 3-disulfide intermediates, are bound relatively tightly, however (Hayer-Hartl et al., 1994; Murai et al., 1995)—the differences of recognition may relate to the amount of hydrophobic surface exposed. Regardless, it appears, as with the pathway studies, that intermediates that have already attained significant secondary and tertiary structure can also be recognized. An extreme of this, perhaps, is a native-like unfolding intermediate of an oxidized $F_{ab}$ molecule, which appears to maintain its native β-sheet contacts and even basic quaternary interactions upon binding transiently to GroEL (Schmidt & Buchner, 1992; Lilie & Buchner, 1995). Here also, a rapidly forming and differently structured early intermediate can be detected bound to GroEL when refolding is initiated from a more fully unfolded state, suggesting that GroEL recognizes multiple different conformations (Lillie & Buchner, 1995).

*c. Conformations while stably bound at GroEL.* A variety of studies have been performed probing the substrate conformation in stable binary complexes between polypeptides and GroEL. Such complexes, formed by dilution of the substrate protein from denaturant, are productive of the native state when ATP and GroES are added. Early work showed that substrate polypeptide in these complexes was exquisitely susceptible to digestion by added protease, suggesting a loosely packed, accessible structure of the bound intermediate (Martin et al., 1991; Mendoza et al., 1992a). Fluorescence studies suggested that tryptophan side chains were present in environments that were less polar than in the fully unfolded state, but more polar than when fully buried in the native state (Martin et al., 1991; Mendoza et al., 1992a; Hayer-Hartl et al., 1994; Weissman et al., 1994; Zahn & Pluckthun, 1994). Binding of ANS to polypeptide–GroEL complexes also suggested the presence of exposed hydrophobic surface (Martin et al., 1991; Mendoza et al., 1992a; Hayer-Hartl et al., 1994).

Deuterium exchange experiments conducted on binary complexes, probing the protection of amide protons in the substrate protein as a reflection of the presence of hydrogen bonded secondary structure, have also provided important insights. One study of bound cyclophilin failed to observe any protection, but its sensitivity would not have allowed detection of protection factors much below 1,000, which are typical of all but later folding intermediates (Zahn et al., 1994b). Deuterium exchange experiments on scrambled 3-disulfide α-lactalbumin intermediates bound to GroEL, monitored by mass spectrometry of the exchanged reaction mixture, revealed a low degree of overall protection of amide protons in the α-lactalbumin, on the order of 2–10 (Robinson et al., 1994). This suggested that unstable secondary structure might be present in the bound protein. A further exchange/mass spectrometry study has been performed more recently on thermally unfolded mature β-lactamase, observing a higher degree of protection

from exchange in β-lactamase bound at GroEL than in the thermally unfolded protein, free in solution. This was interpreted to indicate that, in contrast with cyclophilin, substantial structure was present in the bound polypeptide. Fluorescence characteristics of the bound form further suggested that it was more structured than the acid molten globule state of β-lactamase (Gervasoni et al., 1996).

A recent study with higher resolution has mapped the amide protons protected from exchange in human DHFR bound to GroEL. Here, the DHFR was recovered from GroEL after exchange by refolding it to its native state, where a large number of amide proton probes were highly protected from further exchange. Two-dimensional NMR spectra obtained from this native material could thus inform about exchange that had occurred while DHFR was bound to GroEL. Significant protection, with factors ranging from 5–50, was observed for a number of amide protons, many mapping to what corresponds to the central β-sheet structure in native human DHFR (Goldberg et al., 1997). It thus seems likely that the central sheet structure is present, in an unstable form, in the bound intermediate. Moreover, because DHFR is a parallel β-sheet structure whose β-strands are derived from distant parts of the primary structure, including the $NH_2$- and COOH-termini, a native-like global topology is also likely to be present as well in the bound intermediate.

*4. Binding constants and rates of association*

The rates of association of the various intermediate folding states with GroEL and their respective binding constants have been estimated in a few cases and show a range of values depending on the intermediate bound, as suggested by the discussion in the previous sections. For example, the earliest, burst-phase intermediate of human DHFR (at 1 μM) associated with 10 μM GroEL within the mixing dead-time (5 ms); this put the lower limit on the second-order rate constant for association at about $10^7$ $M^{-1}$ $s^{-1}$. Because GroEL addition halted folding of the later DHFR intermediates within the mixing time, they must also have associated at a similar rate (Goldberg et al., 1997). Maltose binding protein (MBP) intermediates also associated rapidly with GroEL, with rates of $10^7$ and $>10^8$ $M^{-1}$ $s^{-1}$ for the two parallel folding phases (Sparrer et al., 1996). The interaction of GroEL with unfolded RUBISCO occurred at a similar rate of $3.5 \times 10^7$ $M^{-1}$ $s^{-1}$ (Roy et al., 1992). Two early unfolded intermediates of LDH (from *Bacillus stearothermophilus*) associated considerably more slowly with GroEL, with rate constants of $5 \times 10^5$ $M^{-1}$ $s^{-1}$ (Badcoe et al., 1991). The association rates of small unfolded polypeptides with GroEL have also been estimated, ranging from $>10^8$ $M^{-1}$ $s^{-1}$, approaching diffusion-controlled limits, for barnase (Gray & Fersht, 1993) to $0.2–1 \times 10^6$ $M^{-1}$ $s^{-1}$ for apo-α-lactalbumin in the reduced or oxidized molten globule state, respectively (Murai et al., 1995; Katsumata et al., 1996). It has been suggested that some of the differences in association rates may reflect positive or negative electrostatic contributions from interactions between the strongly negatively charged GroEL molecule and polypeptides that are positively charged, such as barnase, or negatively charged, such as α-lactalbumin. Alternatively, some specificity in binding might also be reflected in these rates.

With respect to binding constants, the observation that a later DHFR folding intermediate and GroEL, both at 1.2 μM concentration, formed a stable binary complex implied a dissociation constant for the complex of less than 10 nM (Goldberg et al., 1997). An analogous calculation has been made for the complex of *E. coli* DHFR with GroEL, with an estimate of <85 nM (Clark et al., 1996). The early unfolded intermediates of LDH formed complexes with GroEL with apparent dissociation constants of ~7 nM; the last, molten globule-like, folding intermediate of LDH bound less well by a factor of 5–6 (Badcoe et al., 1991; Staniforth et al., 1994a). For this substrate, the dissociation constants in the presence of ATP or AMP-PNP were also measured and found to be increased to 120 nM and 70 nM, respectively, for the early forms (Staniforth et al., 1994a). Dissociation constants for GroEL complexes with the MBP intermediates were lower, down to 0.01–0.07 nM, but ATP had a larger effect, increasing them 1,000-fold to 10–40 nM (Sparrer et al., 1996). For barnase, the complex involving the populated folding intermediate had a several-fold lower dissociation constant than that of the fully unfolded state (10 nM) (Zahn et al., 1996b, 1996c), whereas for α-lactalbumin, the reduced disulfide folding intermediate bound almost 1,000-fold better (~1 nM) than the oxidized molten globule state (0.7–1 μM) (Katsumata et al., 1996).

Thus, second-order rate constants of association between GroEL and folding intermediates are often fast and, at physiologic GroEL concentration (~1 μM), may produce binding rates on the same order of magnitude as the rates of formation of early collapsed intermediate folding states (10–1,000 $s^{-1}$). The stability of the complexes formed, reflected in their dissociation constants, seem well-correlated with the ability of GroEL binding to halt, in some cases, or significantly slow, in others, the spontaneous folding reaction. Similarly, dissociation constants below 10 nM are consistent with the ability to isolate and purify some substrate–GroEL binary complexes.

A few measurements of GroES–GroEL binding have also been reported. As expected from the stability of the binary complex, dissociation constants were 0.1–0.2 nM in the presence of ADP, increasing to 17 nM in the presence of ATP (Hayer-Hartl et al., 1995). Interestingly, the association rate to form the complex was lower than those reported for some polypeptide–GroEL interactions (above), varying from $1 \times 10^5$ $M^{-1}$ $s^{-1}$ (Jackson et al., 1993) to $4 \times 10^5$ $M^{-1}$ $s^{-1}$ (Hayer-Hartl et al., 1995), both in the presence of ADP. Finally, dissociation constants for ternary GroEL–GroES–polypeptide complexes that take into account *cis* and *trans* topologies (see below) have not been reported, but the ability to isolate such complexes by gel filtration (Weissman et al., 1995) suggests that their dissociation constants must be submicromolar as well.

*5. Conformational changes in the substrate protein associated with binding by GroEL*

*a. Observations.* Although, from the time of early reconstitution studies, it has seemed clear that GroEL facilitates productive folding, the question has often been raised as to whether this is in part accomplished through an action of unfolding or unscrambling, associated with polypeptide binding. Such an idea was first incorporated into a model by Jackson et al. (1993), who argued that binding could be associated with partial unfolding and that ATP binding and hydrolysis would direct a release step that would give a polypeptide another chance at folding. Subsequent kinetic studies supported the notion that GroEL captured folding proteins at a stage when they were prone to forming reversible aggregates and pulled them back toward a less-folded, unscrambled conformation through the action of binding (Peralta et al., 1994; Ranson et al., 1995). Consistent with this idea, Weissman et al. (1994) observed

the same degree of protease susceptibility in a substrate, rhodanese, after a round of release and rebinding by chaperonin, suggesting that rebinding was associated with return of polypeptide to an original non-native state.

Direct evidence for an action of unfolding associated with binding has been acquired for several proteins. For example, native human DHFR, in the absence of ligand, slowly loses activity and binds to GroEL (Viitanen et al., 1991). Likewise, pre-$\beta$-lactamase inactivates in the presence of GroEL and binds to the chaperonin (Laminet et al., 1990). In each case, addition of ATP and GroES causes the polypeptide to refold to its native, active state. In another example, when rhodanese was translated in an *E. coli* S30 extract and retained at the ribosomes by deletion of its stop codon, the nascent chain was not recognized by GroEL, and the protein proceeded to fold partially, forming a protease-protected $NH_2$-terminal domain of 17 kDa. If the rhodanese was released from the ribosomes by puromycin, it was bound efficiently by GroEL and assumed a conformation that was now completely susceptible to proteolysis, indicating that binding by GroEL was accompanied by at least partial unfolding of the $NH_2$-terminal domain (Reid & Flynn, 1996).

In studies with a much smaller polypeptide, the unfolding of barnase, a 6-kDa RNAse from *Bacillus*, has been examined. Although it does not form a stable complex with GroEL upon dilution from denaturant, its spontaneous folding is slowed by a factor of 400 in the presence of GroEL (Gray & Fersht, 1993; Corrales & Fersht, 1995). By examining deuterium exchange of protected backbone amide protons, native barnase was observed to undergo transient global unfolding at a somewhat greater rate (4–25×) in the presence of catalytic amounts of GroEL, exchanging not only surface-exposed amide protons, but also those normally buried in its core structure (Zahn et al., 1996c). Thus, barnase appears to undergo at least a transient global conformational rearrangement while binding to GroEL.

In contrast, our deuterium exchange study of a larger substrate protein, human DHFR, stably bound to GroEL did not detect global unfolding for this polypeptide (Goldberg et al., 1997). In particular, DHFR in the absence of chaperonin does not contain amide protons that exchange only as the result of global unfolding. The finding that the pattern of protection of GroEL-bound DHFR is similar to that of the native protein suggests that the nature of exchange in the presence of GroEL is the same as in its absence. Therefore, it is unlikely that a different mechanism of exchange, such as global unfolding, is occurring. This cannot, however, exclude regional unfolding actions affecting segments without protected amide protons, perhaps paralleling the unfolding observed with the $NH_2$-terminus of newly translated rhodanese (Reid & Flynn, 1996).

*b. Kinetic versus thermodynamic action of GroEL in mediating unfolding.* Is the putative local unfolding associated with GroEL binding a catalytic action? Or does binding to GroEL simply shift an existing unfolding equilibrium to favor the unfolded, GroEL-recognized state? Several authors have suggested that the latter mechanism, thermodynamic partitioning or coupling, may be responsible for the observed unfolding of proteins by GroEL. For example, both pre-$\beta$-lactamase (Laminet et al., 1990; Zahn et al., 1994a) and human DHFR in the absence of ligands (Viitanen et al., 1991) are unstable, relative to an inactive, less-folded state, even in the absence of GroEL. If this state exposes significant hydrophobic segments recognizable by GroEL, binding can occur to form the more-stable binary complex. Even for an otherwise stable protein, such as mature $\beta$-lactamase, destabilization of the native state by elevated temperature may be sufficient to favor, thermodynamically, a GroEL-bound, unfolded form (Zahn & Pluckthun, 1994; Gervasoni et al., 1996). Recent experiments have examined this problem with a chemically modified form of RNAse T1 that cannot complete its folding and so is trapped in a metastable state in equilibrium with a folding intermediate (Walter et al., 1996). When GroEL is added, the equilibrium shifts toward the less-folded state, which binds to GroEL, but the rate of conversion between states is not affected. It thus appears that GroEL acts here to trap the non-native conformation, rather than increasing the rate of its formation, confirming that thermodynamic partitioning is occurring.

Whether such a mechanism contributes to the normal action of GroEL in binding and sequestering aggregation-prone folding intermediates is less clear (see Fig. 5, below). The three basic requirements for thermodynamic partitioning are that the two polypeptide states (intermediate and misfolded, for simplicity) are close to each other in stability, that the equilibrium between them is facile (i.e., there is little kinetic barrier to their interconversion), and that GroEL binds the intermediate state preferentially. For natural substrates of GroEL or Hsp60, only the last of these clearly seems to be met routinely. The first two are very difficult to evaluate for most polypeptides, because all of the non-native states are generally unstable and transient. In the case of mMDH, it has been

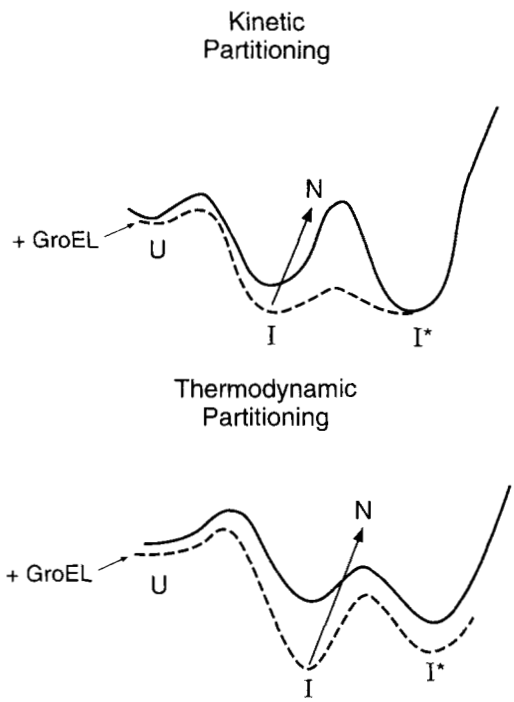


**Fig. 5.** Effects of GroEL on polypeptide folding intermediates. Two reaction coordinate diagrams are shown for the separate models of kinetic partitioning (top panel) and thermodynamic partitioning (lower panel). In both, *U* is the unfolded state, *I* is an intermediate state on the folding pathway, *I** is a misfolded state, and *N* is the native protein; the angled arrow from *I* to *N* is meant to imply folding along another coordinate in three-dimensional space. The solid line indicates the absence of GroEL and the dashed line, its presence. In kinetic partitioning, the major effect of GroEL is to lower the transition state between *I** and *I*, whereas for thermodynamic partitioning, the effect is to increase the stability of *I* relative to *I** (see text for details).

possible to observe both a folding intermediate and a misfolded state early on the aggregation pathway (Ranson et al., 1995). The equilibrium between these states is strongly in favor of the misfolded one, and the rate from misfolded back to intermediate is quite slow; thus, neither of the other requirements of a thermodynamic mechanism appears to be met. Moreover, addition of GroEL increases the rate of the misfolded to intermediate reaction about 500-fold and does so at substoichiometric amounts. These data are consistent with catalysis of unfolding by GroEL and support a kinetic mechanism for its action in assisting mMDH refolding. Likewise, further study of the interaction of barnase and GroEL has indicated that GroEL catalyzes by >1,000-fold the unfolding of native barnase to form a folding intermediate. In contrast, the further unfolding of the intermediate to the fully unfolded state is retarded >2,000-fold in the presence of GroEL (Zahn et al., 1996b). A stable complex between GroEL and the fully unfolded form was observed only at elevated temperature, suggesting that thermodynamic partitioning was operating in this segment of the reaction under these conditions. Thus, both kinetic and thermodynamic mechanisms seem to be operative simultaneously in different segments of the barnase folding pathway.

*c. Crossing the energy landscape in the presence of GroEL.* Such considerations lead to an examination of free energy diagrams for protein folding. Theoretical considerations suggest that a three-dimensional energy landscape represents protein folding better than a classic linear reaction coordinate (Bryngelson et al., 1995). The energy landscape for folding has been described as "rugged" (Todd et al., 1996), at least for substrates that are slow-folding. This implies both that there are kinetic barriers between significantly populated states on the productive pathway, and that there are wells in the landscape that represent kinetically trapped, misfolded states. The size of the barriers and the prevalence of the wells determine whether, how fast, and with what efficiency individual proteins fold spontaneously. The general role of chaperones is often envisioned as *smoothing* this landscape, but a better description is that binding to a chaperone actually *changes* the landscape for that protein. This is particularly true for chaperonins like GroEL, where partially folded forms are bound, unfolding may occur in association with binding, and the substrate becomes encapsulated within the central cavity under GroES. The fact that ATP binding and hydrolysis modulate polypeptide–GroEL interactions adds the further complication of a nonequilibrium process to the analysis. Despite these theoretical problems, it remains possible to visualize a chaperonin-assisted folding reaction with relatively simple reaction coordinate diagrams, as in Figure 5.

Here, we have represented the two extremes of the possibilities for the effects of chaperonin on the rate and yield of a folding reaction described by

$$U \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} I \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} I^* \qquad I \downarrow N$$

where $U$ is the fully denatured state; $I$ is a folding intermediate productive of $N$, the native state; and $I^*$ is a misfolded, nonproductive form. The first diagram illustrates the situation when kinetic effects are paramount. Here, the $I^* \rightarrow I$ barrier is too high to cross to any measurable degree under physiologic conditions ($k_{-2}$ is very small), resulting in a kinetically trapped species ($I^*$). GroEL binding to $I$ and particularly to the $I^* \rightarrow I$ transition state lowers the energy barrier and catalyzes the $I^* \rightarrow I$ reaction, resulting in an improvement in both rate and yield of the folding reaction. In the second diagram, thermodynamic partitioning is operating in the binding of the intermediates—$I$ bound to GroEL is at lower energy than $I^*$, and $k_{-2}$ is relatively large (i.e., the $I^* \rightarrow I$ energy barrier is low enough to permit the reaction to proceed to some extent under physiologic conditions). Therefore, $I$ is more populated in the presence of GroEL, and folding yield is enhanced, even though the microscopic rate constants are unchanged. In reality, GroEL probably influences folding by a combination of these pathways, with a number of $I^*$ species converging on a well-populated, productive folding intermediate. Because of the apparent prevalence of kinetically trapped intermediates in the folding pathways of polypeptides naturally assisted by chaperonins, it seems possible that the first mechanism has a major impact.

The action of GroEL in unscrambling kinetically trapped states in association with repeated acts of binding has been incorporated into a model of "iterative annealing" (Todd et al., 1996), in which GroEL extracts misfolded states from energetic minima through binding and unfolding, then offers the polypeptide a further chance for proper folding through ATP-directed release (Corrales & Fersht, 1996). This seems a further development of the "kinetic proofreading" model of Gulukota and Wolynes (1994). Zahn and his colleagues further suggested that chaperonins have an ongoing annealing function (i.e., local binding/unbinding) within the polypeptide–chaperonin complex, perhaps distinct from active unfolding (Zahn et al., 1996b). Further biochemical data, discussed below, makes it seem possible that encapsulation of the polypeptide under GroES and initiation of folding in that restricted space might produce an even more radical change in the energy landscape than envisioned by these models.

## III. ATP binding and hydrolysis: Driving the reaction cycle

At the heart of GroEL and its reaction cycle are the equatorial ATPase domains, seven per ring, whose binding and hydrolysis of ATP lead to conformational changes in the apical domains that produce high- and low-affinity states for polypeptide substrate. In the absence of GroES, GroEL has been observed to exhibit asymmetry with respect to nucleotide binding (Gray & Fersht, 1991; Bochkareva et al., 1992; Jackson et al., 1993). The structural basis for this asymmetry has been described recently at the level of resolution of electron microscopy by Saibil and coworkers (Roseman et al., 1996), who have shown that binding of ADP and ATP leads to a progressive clockwise twisting (looking down the sevenfold symmetry axis) of the apical domains in one ring, whereas those in the other contract inward. The twisting appears to move the hydrophobic peptide binding sites from a position facing the central channel to one that is turned almost 90° away from the unliganded position. This structural change may account for the reduced affinity of GroEL for unfolded polypeptides in the presence of ATP, as noted in the section above. Under physiologic conditions, however, GroES is present at (at least) equimolar concentration to GroEL, and ATP and ADP are present at 2–10 mM and 0.5–1 mM, respectively. Under these conditions, given that GroES binds rapidly to GroEL in the presence of ATP (Jackson et al., 1993), a dynamic occupation of GroEL with GroES in asymmetric complexes is favored, with *trans*-sided hydrolysis evicting GroES continuously, whereas ADP/ATP exchange presumably regenerates a state with high-affinity for polypeptide (Burston et al., 1995). Thus, at steady-state in the cell, the majority of GroEL molecules will be occupied on one ring by GroES. In electron

microscopy images, GroES binding causes a further major change in the structure of the apical domains of the ring to which it is bound, rotating them upward to contact the GroES mobile loops and continuing the twisting motion as well. The obvious consequences of such nucleotide-driven, GroES-stabilized movement of the apical domains are simultaneously to drive a substrate polypeptide off the apical binding sites into the central cavity to initiate folding (see next section) and to expose it to an entirely new environment in terms of channel walls that are no longer hydrophobic, but likely hydrophilic, in nature. This change of environment presumably favors burial of hydrophobic patches on the substrate bound originally by the apical sites, supporting production of the native state.

Given these considerations, it might seem that GroEL could function as a single-ring machine, but, in fact, both rings are required to drive the complete reaction cycle. Although polypeptide binding and active folding can occur under GroES on one ring in the presence of ATP, discharge of the ligands does not occur (Weissman et al., 1996). The elegant single-turnover experiments of Lorimer and coworkers (Todd et al., 1994) established that release of GroES requires the second ring, being driven by the binding and "quantized" hydrolysis of seven ATPs in this ring, which transmits a signal to the *cis* ring, allosterically, that leads to release. Recent electron microscopy studies (Roseman et al., 1996) suggest that such release may be promoted through further twisting of the apical domains, releasing contact of the remaining hydrophobic binding sites of GroEL from the mobile loops of GroES. Such quantized action highlights the notion that the rings of GroEL appear to function as single units in nucleotide binding and hydrolysis, reflecting strong positive cooperativity within a ring. The two rings are negatively cooperative with respect to each other (Yifrach & Horovitz, 1995, 1996), however, thus favoring binding of ATP to only one ring at a time. This, in turn, dictates asymmetric GroES binding to the nucleotide-occupied ring. The structural bases for both positive and negative cooperativity remain unclear, although in the former case, contacts within a ring, between equatorial domains, and between intermediate domains and neighboring apical domains, appear to be important; in the latter case, presumably the two major sites of contact across the equatorial plane between each subunit and its neighbors in the opposite ring must provide the signaling pathway (Braig et al., 1994; Roseman et al., 1996).

## IV. GroEL–GroES–polypeptide ternary complex: The folding-active *cis* complex

### *A. Cis and trans ternary complexes*

A variety of experiments, both in vivo and in vitro, have made it clear that the productive folding of substrate polypeptides, at least under nonpermissive conditions where unassisted folding does not occur, requires GroES as well as GroEL (e.g., Schmidt et al., 1994a), implying that ternary complexes must exist. The nature of such complexes was intimated from elegant cryo-electron microscopy studies of Saibil and coworkers (Chen et al., 1994). Most strikingly, they observed that, when GroES became bound to GroEL, the apical domains of GroEL were opened upward and outward, nearly doubling the size of the central channel under GroES. This raised the question of whether a polypeptide could fit entirely inside this space underneath GroES. Such complexes can indeed be formed in vitro in the presence of ADP, as demonstrated by order-of-addition experiments, using both susceptibility to subsequently added protease and hit-and-run crosslinking by substrate of proteolytically marked GroEL rings (Weissman et al., 1995). Under conditions where approximately equimolar GroEL, GroES, and an unfolded substrate polypeptide such as rhodanese are combined, about half of the ternary complexes are *trans*, with GroES and polypeptide occupying opposite rings, and the other half are *cis*, where non-native polypeptide and GroES are bound to the same ring. This suggests that there is no strong kinetic bias toward the formation of either species.

A specific role for *trans* complexes is not clear. Larger proteins, such as the 68-kDa phage P22 tailspike protein or the 75-kDa methylmalonyl-CoA mutase, appear to be able to form only *trans* complexes, presumably because they cannot fit underneath GroES in a *cis* configuration (Brunschier et al., 1993; Gordon et al., 1994; Weissman et al., 1995). Whether the folding of such proteins can be assisted by GroEL is still an open question. For smaller polypeptides such as ornithine transcarbamylase (OTC, 36 kDa), however, single-turnover experiments, described more fully below, show that the *trans* complex is ineffective in producing the native state (Weissman et al., 1995). Whether non-native forms are released from the *trans* configuration has not been established fully, although some results suggest that nonproductive release can occur in vitro (Mayhew et al., 1996; W.A. Fenton, unpubl.). In the cell, release from a *trans* complex presumably gives a polypeptide a chance to rebind to GroEL or other chaperones for further attempts at folding, or to be recognized by proteases for degradation. For proteins greater than 60–70 kDa in size, which can bind to GroEL, but are unable to fit under GroES to form *cis* ternary complexes, such release would be critical to prevent GroEL from becoming engorged with unfoldable proteins. Whether the folding of such larger proteins can be assisted by GroEL, either by unscrambling those portions of their structures associated with the GroEL binding sites or through an action of orchestrated release, is unknown and an active area of study both in vivo and in vitro.

### *B. Symmetric complexes*

Another possible form of a GroEL–GroES–polypeptide complex has been suggested by observations in the electron microscope of symmetric complexes, called "footballs," which have GroES molecules bound to both ends of the GroEL cylinder, formed when GroEL and GroES are incubated in certain near-physiologic combinations of buffer and adenine nucleotide (Azem et al., 1994; Schmidt et al., 1994b). Although their formation has been correlated with more efficient polypeptide folding (Azem et al., 1995; Diamant et al., 1995; Llorca et al., 1996), their role in the reaction remains unclear (e.g., Hayer-Hartl et al., 1995). Titration experiments, measuring either GroES binding (Langer et al., 1992; Jackson et al., 1993; Engel et al., 1995) or the inhibition of the ATPase activity of GroEL by GroES (Todd et al., 1994) have usually shown a 1:1 stoichiometry of GroES to GroEL. In addition, under conditions favoring footballs when only GroEL and GroES were present, unfolded DHFR and rhodanese both prevented their formation (Engel et al., 1995). Furthermore, preformed *cis* ternary complexes containing non-native OTC produce a high yield (>80%) of native enzyme in a single turnover when discharged by ATP without additional GroES (Weissman et al., 1995) (see below). These data all argue that footballs are not *obligatory* in the folding reaction. The hypothesis that they can play a *transient* role as an intermediate form in moving from *trans* to *cis* remains attractive,

however, because it would appear to be an efficient means to achieve the *cis* state (see Fig. 4). The football form would be expected to be highly dynamic and unstable in the presence of ATP, whose hydrolysis causes GroES discharge from the opposite ring. This could account for the necessity for chemical crosslinking in order to detect such complexes in vitro during a folding reaction (e.g., Azem et al., 1995). Overall, the question in functional terms is whether polypeptides bound in *trans* depart GroEL before they can be capped by an incoming GroES. Recent experiments, examining the effect of polypeptide trap mutants of GroEL on the folding of OTC from preformed *trans* ternary complexes, suggest that a substantial fraction of the *trans*-bound polypeptide is released in a non-native form that can bind to a trap, even in the presence of excess GroES (W.A. Fenton, unpubl.). This argues that few symmetric complexes containing bound polypeptide are being formed under these conditions.

### C. *The folding-active intermediate of a chaperonin reaction: Cis ternary complex*

Although the roles of *trans* complexes and footballs appear to be at most as intermediates on the pathway to the folding-productive state, a number of experiments have demonstrated that the *cis* complex is central to the folding reaction. Because stable ternary complexes can be formed in the presence of ADP and isolated, we have been able to address the question of whether *cis* or *trans* ternary complexes are productive of native forms (Weissman et al., 1995). This was facilitated particularly by the availability of a substrate, OTC, that is folded efficiently during a single round of binding and release from GroEL. *Trans* complexes were prepared in ~100% yield by first forming asymmetric GroEL–GroES binary complexes in the presence of ADP, then adding OTC diluted from acid. *Cis* complexes were formed by the opposite order of addition, with polypeptide added first, then GroES. Then, because ~50% of these ternary complexes would be expected to be *trans*, polypeptide was removed from the *trans* form by protease treatment, under conditions in which polypeptide bound in *cis* complexes was protected from proteolysis. In kinetic studies, when *cis* ternary complexes were incubated with MgATP for periods ranging from 15 s to 15 min, followed by apyrase treatment to quench further folding, assembly-competent OTC was produced rapidly ($t_{1/2}$ < 15 s) and extensively (>80% yield); by contrast, *trans* complexes exhibited slower kinetics ($t_{1/2}$ = 5 min), but ultimately full recovery. Production of native OTC from *trans* was further slowed by making GroES limiting in concentration. These observations suggested that *cis* complexes were productive, whereas *trans* complexes might not be, with activity produced by their conversion to *cis* after an initial round of ATP hydrolysis, GroES release, and GroES rebinding.

Support for such a hypothesis came from a single-turnover study, made feasible by the characteristics of a mutant GroEL, called SR1, a single-ring version of GroEL designed with information from the unliganded crystal structure. Each GroEL subunit forms four major contacts across the equatorial plane with the opposite ring (Braig et al., 1994). These residues were changed simultaneously to alanine, resulting in the generation of a stable heptamer (single-ring) version of GroEL. The mutant complex was observed to bind polypeptide and GroES with the same efficiency as wild-type GroEL but, strikingly, SR1 could not release GroES even in the presence of MgATP (Weissman et al., 1995). This behavior was consistent with the studies of Todd, Lorimer, and coworkers (Todd et al., 1994), observing that *trans*-sided ATP hydrolysis (i.e., hydrolysis in the ring opposite that bound by GroES) was required for release of GroES—because SR1 lacks a *trans* ring, no signal can be transmitted for GroES release. Thus, SR1 could function as a trap for GroES, capturing GroES after its initial release from a ternary complex and confining the reaction to one turnover. The results of the single-turnover study of OTC refolding from wild-type GroEL in the presence of SR1 were unambiguous: *cis* ternary complex produced native OTC rapidly and extensively, even in the presence of SR1, whereas *trans* complex failed to produce any significant activity (Weissman et al., 1995).

### D. *Role of the cis space in the folding reaction*

The question of what happens in a *cis* ternary complex to facilitate productive folding has become crucial to understanding the mechanism of GroEL-assisted folding. Important steps toward an answer have been to resolve what initiates folding in these complexes and to examine how far folding proceeds before discharge of the ternary complex occurs, triggered by *trans*-sided ATP hydrolysis and release of GroES. To observe initiation of polypeptide folding inside GroEL complexes, we utilized fluorescence anisotropy, which reports on the local rotational flexibility of a fluorophore, and, in this context, could give an indication of the overall flexibility of the polypeptide chain. The monomeric substrate protein, rhodanese, was labeled on its four cysteines with pyrene-maleimide, and binary complexes were formed between unfolded rhodanese and either wild-type GroEL or the single-ring SR1 mutant. The anisotropy of the bound rhodanese was elevated, consistent with its interaction with GroEL. Various nucleotide additions were then made, in the absence or presence of GroES. A striking drop in anisotropy, indicating an increase in polypeptide flexibility, was observed only when MgATP and GroES were added to either binary complex (Weissman et al., 1996). In particular, neither ATP alone nor GroES and ADP could trigger a significant drop of anisotropy. This requirement for both ATP and GroES exactly parallels the requirement for these additions for productive folding. Significantly, the drop in anisotropy with GroES and ATP occurred with a $t_{1/2}$ of ~1 s, a time much shorter than the $t_{1/2}$ of ~15–30 s for ATP hydrolysis and concomitant GroES discharge from wild-type GroEL. These observations indicated that rhodanese was released rapidly by ATP from its binding sites in the central cavity under GroES in the *cis* ternary complex and implied that folding commenced rapidly in *cis* ternary complexes in the presence of ATP.

To assess the extent to which folding of a monomeric protein could proceed under GroES in the presence of ATP, the enzymatic activity of rhodanese was measured after GroES and ATP were added to an SR1-rhodanese binary complex and the complex reisolated by rapid gel filtration (Weissman et al., 1996). Strikingly, rhodanese activity was recovered with the SR1-ES complexes. This indicated that not only the initiation of rhodanese folding, but also its completion to the native state, could occur in the *cis* ternary complex. The kinetics of refolding were remarkably similar to those at wild-type GroEL, a surprising result considering the differences between the two reactions. For example, in the case of SR1, folding is confined inside the SR1-GroES cavity and is associated with a single round of ATP hydrolysis (leaving non-exchangeable ADP in the SR1 ring), whereas with wild-type GroEL, the reaction occurs with many rounds of release and rebinding of both GroES and rhodanese (see below), associated with multiple

rounds of ATP hydrolysis. Because of the possibility that SR1, with its multiple mutations, might not reflect physiological behavior of wild-type GroEL accurately, the same type of experiment was performed with wild-type GroEL, preventing release of GroES by employing the nonhydrolyzable ATP analogue, AMP-PNP. As with SR1, rhodanese molecules trapped underneath GroES on GroEL reached a native, active form that remained associated with the GroEL complex, as observed by assay of gel filtration fractions.

A further question is whether the *cis* cavity underneath GroES plays an active, rather than purely passive, role in directing folding of the sequestered polypeptide. In an attempt to address this point, green fluorescent protein (GFP) was unfolded and bound to SR1, where it was not fluorescent (Weissman et al., 1996). Upon addition of GroES and ATP, fluorescence reappeared, suggesting that the GFP had refolded, and more than 60% of the fluorescent protein could be recovered with SR1 in gel filtration. The fluorescence characteristics of the sequestered, refolded GFP were identical to free GFP in solution, except for the rate of decay of fluorescence anisotropy, which was retarded significantly for the bound protein (correlation time of 54 ns, corresponding to a species of ~120 kDa, versus 13.2 ns for free GFP). This suggests that GFP is not freely tumbling in the *cis* space, implying a continuing interaction of the folded protein with the walls of the cavity. Such interactions would likely have a significant impact on less-stable and probably less-compact folding intermediates in the course of the refolding. In addition, even without specific participation of the cavity surface, simply sequestering the polypeptide in a relatively small volume may serve to alter the spectrum of accessible folding intermediates or the folding pathway. In fact, theoretical studies suggest that confinement alone could produce stabilization of secondary structures (e.g., Yee et al., 1994). How much this contributes to the folding of any individual protein remains unclear.

### *E. Folding governed by a "timer" mechanism*

Although the results of the SR1 experiments described above indicate that rhodanese (and GFP) can complete refolding underneath GroES if the GroES is prevented from release, they must be interpreted in light of the normal physiology of GroEL–GroES reactions. In the presence of two rings and hydrolyzable ATP, as in the cell, hydrolysis of ATP in the ring in *trans* to GroES would trigger its release after 15–30 s (Todd et al., 1994; Burston et al., 1995; Hayer-Hartl et al., 1995). Thus, in contrast to the foregoing studies where the folding-active intermediate state has an essentially infinite lifetime, under normal conditions, polypeptide has only 15–30 s in the folding-active complex. With discharge of GroES, polypeptide can also be discharged, leaving in any of three states: a native state, if folding has been completed; a state committed to reaching native form; or an uncommitted, non-native state that can partition between misfolding, aggregation, or binding to chaperones, including another GroEL molecule. The folding-active state of the chaperonin reaction is thus normally governed by a timer whose set-point must apparently accommodate the needs of the cell; that is, the set-point must be sufficiently long to permit a reasonable percentage of many polypeptides to reach a native or committed state before the timer goes off, but not so long that it unnecessarily prevents folded or committed proteins from departing the cylinder. This would be of particular importance for oligomeric proteins, whose departure would be required to reach a final, assembled, active state. The in vitro studies preventing GroES release, that is, setting the timer to "infinity," are thus not an accurate reflection of the normal cycle, where the consequence for at least some proteins, such as rhodanese, mMDH, or RUBISCO, is that only a small fraction reach a committed or native state in a single normal lifetime of a *cis* ternary complex. The fate of the remaining non-native molecules is release from GroEL into the bulk solution with rebinding and further rounds at GroEL required for reaching native form.

### *F. Release of non-native polypeptides during the GroEL–GroES reaction*

A basic question raised about the chaperonin reaction concerns the level of commitment a polypeptide achieves during a reaction cycle. If there were full commitment, the chaperonin should release into solution only fully folded native forms. Alternatively, there might be less than full commitment, and non-native forms would be released during the reaction. The production of so-called trap mutants, a class of GroEL mutant that can bind polypeptides but not release them, even in the presence of GroES and ATP (Fenton et al., 1994), allowed an early test of commitment (Weissman et al., 1994). In particular, if non-native forms were being released during the chaperonin reaction, then addition of an excess of a trap mutant should capture these forms and block their refolding, reducing the yield of native form. By contrast, if the polypeptide were fully committed, trap mutants would not affect efficiency of recovery of the native state. To our surprise, the reaction was not fully committed: addition of only a 2:1 molar excess of trap molecules to a GroES- and ATP-mediated folding reaction from a GroEL–rhodanese binary complex reduced recovery of rhodanese activity to a level of ~15–20%. In addition, physical transfer to the trap was observed. This occurred rapidly, with ~50% of molecules transferring in 30 s and 80% by 2 min. During these same times, in the absence of trap, only 5% and 10% of the rhodanese activity was recovered. Thus, the rate of release of non-native forms is nearly 10-fold the rate of production of the native, active form.

In a second test, conducted at about the same time, Todd, Lorimer, and coworkers also observed release of non-native forms from GroEL, using as substrate the subunit of the homodimeric RUBISCO from the cyanobacterium *R. rubrum* (Todd et al., 1994). Radiolabeled RUBISCO subunits in a binary complex with GroEL were incubated with a nonlabeled, metastable folding intermediate of the subunit, and the amount of label remaining in the complex was quantitated after gel filtration. In the presence of ATP and GroES in the incubation mixture, a rapid fall in the level of radiolabeled protein bound to GroEL was observed, exceeding any conversion of the labeled input material to native form. This indicated that the bound protein must be exchanging with the nonlabeled folding intermediate in solution, that is, that it left GroEL in non-native form.

Since these initial studies, several other groups have reported observing departure of a fraction of protein in non-native form from chaperonin during the reaction (Ranson et al., 1995; Smith & Fisher, 1995; Taguchi & Yoshida, 1995). Furthermore, the question of whether *cis* ternary GroEL–GroES–polypeptide complexes can release non-native species along with native forms has also been addressed experimentally (see above for discussion of *trans* complexes). A mixed-ring GroEL complex, MR1, which can only bind polypeptide and GroES on one and the same ring, was designed. This was accomplished by dissociation and reassembly of rings in a mixture of wild-type GroEL and a mutant defective in both

polypeptide and GroES binding (carrying apical-domain mutations) (Burston et al., 1996). A characteristic migration in anion exchange allowed purification of mixed ring complexes containing one mutant ring and one wild-type ring. Notably, the mutant ring had an unperturbed ATPase activity and thus should carry out normal signaling to the wild-type ring. We observed that MR1 refolded rhodanese with the same kinetics as wild-type and that, as with wild-type, addition of polypeptide traps quenched recovery of activity to a level of ~10%. Because MR1 is a *cis*-only, double-ring complex, the non-native forms of rhodanese that were trapped during the reaction must have been released from a *cis* configuration.

What is the purpose of releasing non-native forms? Why doesn't the reaction achieve full commitment for its substrates? Several purposes seem to be served:

1. Mutant proteins or proteins damaged by proteolysis, heat stress, or oxidation may be unable to reach native form and are liable to misfold. If misfolded forms became bound by GroEL, they would likely remain there, tying up the chaperonin irreversibly, unless there was a release mechanism. The release of non-native forms from GroEL permits such species to partition kinetically in solution in the cell, ultimately reaching other chaperones or the proteolytic machinery that can dispose of them (e.g., Kandror et al., 1994).

2. Similarly, there are likely to be proteins whose folding intermediates can be recognized by GroEL but not productively folded by it. This has been demonstrated for several heterologous proteins, not normally substrates for GroEL-mediated folding, for example, actin and tubulin subunits (Tian et al., 1995). More importantly, the same situation may hold for a fraction of *E. coli* proteins, exposed to GroEL in the bacterial cytoplasm and able to bind to it, but not productively folded in *cis* complexes. Such proteins must necessarily depart the chaperonin, or they would engorge GroEL. In an in vitro model of this situation, using a nonhomologous substrate, Bukau and coworkers have shown that the monomeric protein, luciferase, is bound efficiently by GroEL, but cannot be released from it productively (Schroder et al., 1993). On the other hand, they observed that the *E. coli* Hsp70 system, DnaK-DnaJ-GrpE, can direct the protein to native form. Luciferase bound initially at GroEL was shown to transfer efficiently to polypeptide trap mutants of GroEL when GroES and MgATP were added, demonstrating release of non-native protein. Most importantly, if, rather than adding the traps, DnaK, DnaJ, and GrpE were added, luciferase was refolded efficiently to native form (Buchberger et al., 1996). Thus, in this model system in vitro, kinetic partitioning to another chaperone permits refolding of a substrate that is not able to be assisted by GroEL.

3. In some other cases, release of non-native forms from GroEL may enable productive folding that otherwise would not occur. This certainly seems likely for proteins too large to fit inside the *cis* space, that can only be bound in *trans* (e.g., phage P22 tail spike protein; Galisteo et al., 1995). Based on the lack of folding of even a small protein, such as OTC, bound in *trans* (Weissman et al., 1995), it seems likely that larger unfolded proteins bound in this location will not be released productively from GroEL, although binding to GroEL may act locally to unfold or stabilize such intermediates, preventing aggregation. Here also, to keep GroEL available for productive folding, these larger proteins would be released from the *trans* position in an uncommitted state to partition among other components.

### *G. Release of both native and non-native forms under physiologic conditions*

Some investigators have suggested that the release of non-native forms reflects "leakiness" that occurs under in vitro conditions and that the high concentrations of macromolecules, present under physiological conditions, would act to exclude solvent and reduce diffusion rates, disposing to a fuller level of commitment in vivo (Ellis & Hartl, 1996). This point of view would seem to ignore the results from in vitro studies where both the rate and extent of release of non-native forms are well beyond "leakiness." For example, the rate of release of non-native rhodanese is 10 times the rate of appearance of the native active form, and the extent of transfer to trap is 50% of the molecules in 30 s (Weissman et al., 1994). Although it is not clear whether every rhodanese molecule leaves chaperonin with each pair of rounds of ATP hydrolysis (see Fig. 4; note that the first hydrolysis occurs in the *cis* ring, associated with GroES binding; the second hydrolysis in the *trans* ring, with GroES departure), a substantial fraction clearly leaves with each cycle.

Moreover, the question of release of non-native forms under physiologic conditions has been addressed directly (Burston et al., 1996). Rhodanese–GroEL binary complexes were formed and either added to undiluted *Xenopus* oocyte extracts supplemented with GroES and ATP, with or without a trap, or injected directly into intact oocytes with the same additions. As with the in vitro experiments, we once again observed that addition of trap molecules largely abolished recovery of activity.

Although the oocyte cytoplasm does not contain the identical constituents of the bacterial cytoplasm where GroEL functions, its overall concentration of macromolecules must be very similar. To exclude the possibility that some sort of "commitment factor" might be present in *E. coli* but absent from the eukaryotic cell, we have also recently examined whether partitioning to traps occurs in bacterial S30 translation mixtures (S.G. Burston, unpubl.). Rhodanese molecules, translated in this mixture, were observed to become associated with GroEL rapidly. Addition of a polypeptide trap, along with GroES and MgATP, produced inhibition of folding and extensive transfer to the trap, thus indicating that, at least in a two- to threefold diluted bacterial extract, the same release of non-native forms occurs.

### *H. A role for ATP binding, as well as hydrolysis, in the folding cycle*

As our anisotropy studies with rhodanese reflected, polypeptide bound at GroEL commenced folding rapidly in the presence of ATP and GroES ($t_{1/2}$ = 1 s), well before ATP hydrolysis and release of GroES occurred ($t_{1/2}$ = 15–30 s). This effect was observable both with wild-type GroEL and with SR1, so it seemed that the act of binding GroES and ATP might be sufficient to initiate productive folding. By contrast, in the presence of ADP, despite the fact that GroES could be bound to form a *cis* ternary complex, no drop of anisotropy was observed, reflecting that rhodanese was not folded efficiently in the presence of ADP and GroES (Weissman et al., 1996). Recent cryo-electron microscopy studies of GroES–GroEL complexes in the presence of nucleotides provide some insight into these results (Roseman et al., 1996). The

GroEL apical domains, which open upward and outward in the presence of ADP, are subject to further movement in the presence of ATP. Whereas the polypeptide might still cling to a remaining patch of exposed apical peptide binding surface in ADP, the further twisting of the apical domains in the presence of ATP would now turn the peptide binding surface fully away from the central channel, leading to complete release of polypeptide. Based on the results with SR1, it seems likely that ATP binding in the *cis* ring of wild-type GroEL can accomplish this. Binding of ATP in the *trans* ring, however, may also be able to drive the conformational change that promotes the rapid commencement of folding in *cis*, as shown by the single-turnover studies in which OTC was discharged from *cis* ternary complexes formed in the presence of ADP. Additional studies seem necessary to clarify the different roles of ATP binding and ATP hydrolysis in each of the rings in promoting folding in *cis* ternary complexes and driving the chaperonin cycle.

## V. Concluding remarks

The chaperonin machinery, exemplified by GroEL/GroES in eubacteria and Hsp60/Hsp10 in eukaryotic organelles, clearly plays an essential role in protein biogenesis and cellular homeostasis. It has evolved to cope not only with a wide variety of substrates, but also with changing conditions of cellular stress. Therefore, it is both simple in concept (for example, binding collapsed folding intermediates with exposed hydrophobic surfaces) and complex in execution (for example, differentiating by orders of magnitude between different non-native, compact folding intermediates of the same polypeptide). Although the simple outlines seem to be in place and some of the complexities have yielded to structural and biochemical investigation, many aspects remain obscure. This is particularly true in the areas of the nature of polypeptide binding and concomitant unfolding, the role of structural changes in the *cis* GroEL–GroES–polypeptide ternary complex in initiating folding, and the active participation of the *cis* cavity in directing productive folding within it. Each of these is the subject of active investigation and may soon offer new insights into this vital cellular function.

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