Putting a lid on protein folding: structure and function of the co-chaperonin, GroES

Wayne A Fenton[1], Jonathan S Weissman[2] and Arthur L Horwich[2]

The co-chaperonin GroES is an essential partner in protein folding mediated by the chaperonin, GroEL. Two recent crystal structures of GroES provide a structural basis to understand how GroES forms the lid on the folding-active *cis* ternary complex, and how the GroEL–GroES complex enhances folding.

Addresses: [1]Department of Genetics and [2]Howard Hughes Medical Institute, Yale School of Medicine, 333 Cedar Street, New Haven, CT 06520, USA.

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Two recent papers provide us with a first look at atomic resolution at the structure of the co-chaperonin molecule, GroES or cpn10, that participates with GroEL in mediating protein folding. The first paper, from Hunt, Deisenhofer and coworkers [1], presents the structure, refined at 2.8 Å, of GroES from *Escherichia coli*; the other, from Hol and coworkers [2], presents the structure at 3.5 Å of the GroES homolog (cpn10) from *Mycobacterium leprae*.

These structures have appeared at a most propitious time, when concurrent functional studies are providing a new understanding of how this essential partner of GroEL participates in ATP-dependent protein folding. These functional studies are outlined below, followed by a discussion of the structural findings and how they relate to current models for the mechanism of GroEL–GroES-mediated protein folding.

Functional analyses – GroES is a full partner in the folding reaction

GroEL and its immediate chaperonin relatives are large tetradecameric complexes composed of two back-to-back seven-membered rings of 58-kDa subunits [3]. These complexes bind non-native proteins in a central channel [4,5] and have a broad and essential role in facilitating protein folding *in vivo* [6,7]. Studies have demonstrated that full function of GroEL is dependent on the presence of the co-chaperonin, GroES, a smaller, seven-membered ring of 10-kDa subunits that binds at one or both ends of the GroEL cylinder in the presence of adenine nucleotides [8–13]. GroES is essential for bacterial growth under all conditions *in vivo* [14] and seems to be required for productive folding of many if not all of the protein substrates of GroEL. Moreover, for proteins for which overexpression of GroEL increases the yield of native protein *in vivo*, co-overexpression of GroES is also required [15,16]. Similarly, under conditions *in vitro* where a protein cannot fold to its native form spontaneously, productive folding by GroEL requires the cooperating action of GroES [17]. Even for proteins able to fold spontaneously, GroEL-driven folding in the absence of GroES seems to be qualitatively different from that in its presence. For example, early studies indicated that, in the presence of GroES, chicken dihydrofolate reductase appeared to undergo at least partial folding in association with GroEL, whereas, in its absence, folding occurred exclusively in solution [18].

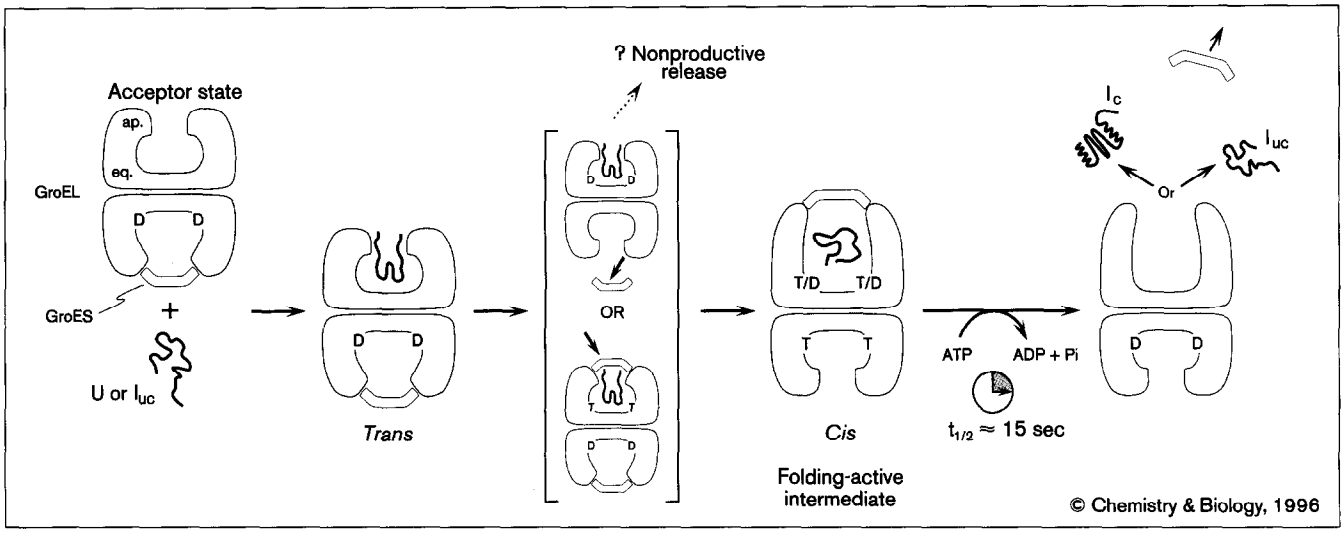
GroES binding produces a chamber

Several recent observations provide insight into the mechanism by which GroES alters a GroEL-dependent folding reaction (Fig. 1). GroES associates with GroEL in the presence of adenine nucleotides, binding at one (or both) ends of the GroEL cylinder. In the presence of ATP, this association is dynamic, as ATP hydrolysis in the GroEL ring not occupied by GroES triggers quantitative release of GroES [19]. How does this dynamic association of GroES with GroEL work to assist protein folding by GroEL? One effect of GroES binding is to increase the cooperativity of ATP binding and hydrolysis by GroEL, so that ATP hydrolysis occurs simultaneously in the unoccupied ring in a 'quantized' fashion [19–22]. Because polypeptide release and folding is dependent on ATP binding and hydrolysis, this increased cooperativity could have a critical role in coordinating the concerted and productive release of polypeptide from its binding sites on GroEL.

The second major effect of GroES binding is to induce dramatic conformational changes in GroEL. Cryoelectron microscopy studies reveal a wholesale conformational change of the GroEL ring to which GroES binds, involving an opening upward and outward of the GroEL apical domains to make contact with GroES and producing an approximate doubling of the volume of the GroEL central channel underneath GroES [23]. The possibility that a polypeptide could occupy such a space was first intimated by an early study showing that a GroEL-bound polypeptide could be crosslinked to GroES [24]. More recently, it has been shown that polypeptides can become efficiently bound in this space during a folding reaction [25]. Strikingly, for at least one substrate, ornithine transcarbamylase (OTC), productive release from GroEL requires localization in this space under GroES (the *cis* configuration), with no folding observable from the *trans* complex, where polypeptide is bound to the ring opposite that bound by GroES.

Under physiologic conditions, non-native polypeptide is probably initially bound by an asymmetric GroEL–GroES complex in the accessible central

Figure 1



Model for a GroEL–GroES-mediated folding reaction. The asymmetric GroEL–GroES complex (first panel; ap., apical domain; eq., equatorial domain; D, ADP bound) is probably the polypeptide acceptor state *in vivo*; it 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 [19,26]; two of the possible pathways of GroES release and rebinding that lead to the *cis* complex are shown (third panel; T, ATP bound; see text). 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 [25,33]. Simultaneously, ATP binding in the *trans* ring starts the timer for hydrolysis and release. When ATP hydrolysis occurs in the *trans* ring ($t_{1/2} \approx 15$ sec), GroES is released [19,27], giving the polypeptide the opportunity to depart (last panel). The released polypeptide is either committed to fold (or already folded) (I_c) or 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 upon ATP/GroES binding and release.

channel, in *trans* to GroES (Fig. 1). During the folding reaction, however, GroES must become bound to the other side to produce the productive *cis* ternary complex. Three distinct mechanisms have been proposed for the conversion of the ternary complexes from *trans* to *cis* topology. In one, polypeptide binding alone induces release of nucleotide and GroES, allowing GroES rebinding in *cis* [26]. Recent studies with two well characterized substrates, rhodanese and OTC, indicated, however, that polypeptide binding to preformed binary GroEL–GroES complexes in the absence of ATP hydrolysis resulted in little (< 3 %) *cis* complex formation [25], making this mechanism less likely. In a second proposed mechanism, ATP hydrolysis in the *trans* ternary complex induces quantitative release of GroES [19]. GroES can then rebind to form a *cis* complex with an efficiency of ~50 % [25]. As a final possibility, a second GroES molecule binds to the *trans* ternary complex to form a 'football' intermediate, with GroES bound at both ends of the GroEL double toroid. While 'footballs' do not seem to be strictly required for productive folding *in vitro* [25,27], recent studies suggest that they might be formed under physiologic conditions [8,12,13]. Moreover, such intermediates could provide an efficient means *in vivo* of converting from *trans* to *cis*, as binding of the second GroES heptamer would have to occur on the same side as polypeptide.

Folding inside the chamber

How much folding actually occurs within the *cis* ternary complex? When OTC is released from such a complex in the presence of ATP, it seems already to be largely committed to achieving its native form [25,28]. Released OTC subunits cannot be bound by another GroEL molecule, implying that they are no longer non-native and suggesting that they acquired this form while associated with or during discharge from GroEL. Yet, for a number of other protein substrates, only a small fraction is committed to folding to the native state in a single release from GroEL; a large percentage is released in non-native forms during a folding reaction, because these intermediates can be rebound by other GroEL molecules [19,29–32]. Multiple rounds of binding and release ('jumping') normally occur before all molecules are folded.

This dichotomy has been recently addressed by studies of rhodanese, a monomeric protein for which a large fraction of released molecules is not committed to fold [33]. When a *cis* ternary GroEL–GroES–rhodanese complex is formed in the presence of ATP, there are changes in the flexibility of bound rhodanese on a timescale of 1–5 s, much shorter than the timescale of ATP hydrolysis, which triggers GroES release ($t_{1/2} \approx$ 15–30 s). This suggests that there are changes in the conformation of rhodanese (i.e., folding) occurring at GroEL, underneath GroES, before

either GroES or polypeptide can leave. Further, when GroES is prevented from leaving GroEL, either in experiments with a single ring mutant of GroEL, which cannot release GroES, or by use of a nonhydrolyzable ATP analog, rhodanese can complete folding to native form while remaining underneath GroES in a ternary complex. Significantly, similar conformational changes are not observed when the ternary complex is formed in the presence of ADP, nor does efficient folding occur from these complexes [33,34].

Thus, it seems that the channel in *cis* ternary complexes is the site where productive folding is initiated upon ATP binding. On the other hand, it is not obligatory for folding to be completed in this space, because ATP hydrolysis acts as a timer and induces GroES release with a half-time of 15–30 s. For some polypeptides, such as OTC, folding may be largely completed within this time [25,33]. More generally, however, only a fraction of bound molecules reach a conformation that is either native or committed to fold before the 'timer' goes off, allowing release into bulk solution. The released polypeptides are rebound by the same or another GroEL molecule, reinitiating the folding cycle. Rebinding of polypeptide might also be important in promoting unfolding of incorrect structures in kinetically trapped intermediates. In support of this proposal, Zahn, Fersht and coworkers [35] recently demonstrated that GroEL can bind the native form of the 12-kDa protein barnase and catalyze its unfolding.

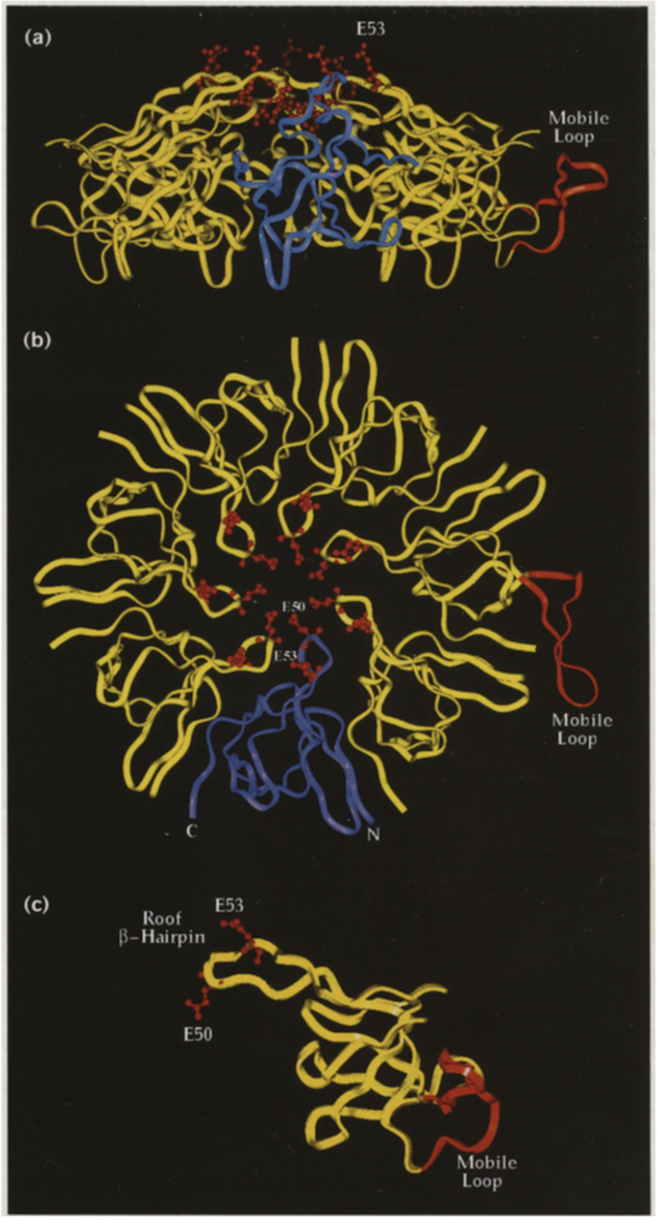
Structural analysis

Viewing GroES in functional terms as a 'lid' on a folding chamber evokes specific questions when looking at its structure at atomic resolution. What does the undersurface of GroES look like? Does this surface make direct contact with polypeptide or with GroEL? What other structures can interact with GroEL or polypeptide?

The cavity in GroES and the GroEL–GroES central channel

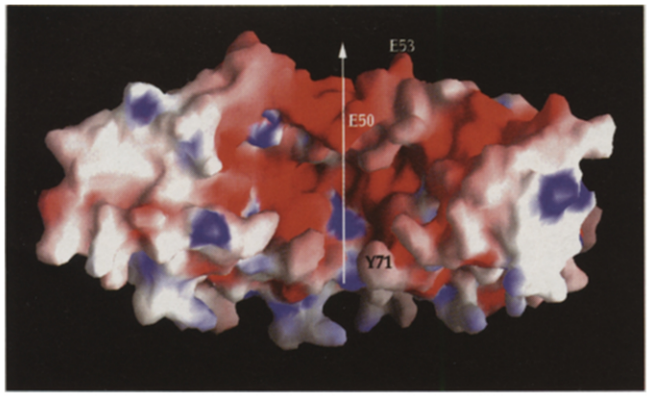
The seven subunits of GroES form a dome-shaped structure about 30 Å high and 70–80 Å in diameter, with an inner cavity 20 Å high and 30 Å in diameter [1,2] (Fig. 2). Each subunit is composed of a body of two orthogonal antiparallel β-sheets, from which project two β-hairpins, one extending radially inward and upward from the top to contribute the 'roof' of the dome structure, and the other probably extending outward and downward from the bottom, although this segment, the 'mobile loop', is resolved in only one of the seven subunits of GroES, by virtue of a lattice contact, and not at all in the cpn10 structure (see below). The interactions between subunits are mediated mainly by hydrophobic contacts between two antiparallel β-strands from the bodies of adjacent subunits. Intriguingly, the tips of the β-hairpins forming the roof contain two or three negatively charged residues from each subunit, resulting in an intense negative potential at the top of the dome. These acidic residues are not highly conserved, however, and are even replaced with basic ones in certain GroES homologs, suggesting that negative charge is not rigorously required in this location, but, rather, that hydrophilicity is conserved.

Figure 2



Structure of GroES. **(a)** Side and **(b)** top orthogonal views of a ribbon diagram of the GroES heptamer. One subunit is shown in blue, and the single resolved mobile loop is shown in red. Also in red are ball-and-stick representations of the roof β-hairpin glutamates, E50 and E53. The amino (N) and carboxyl (C) termini of the blue subunit are indicated in (b). **(c)** The monomer of GroES with the resolved mobile loop. This view is rotated about 45 degrees relative to that in (a) to show the central barrel and roof β-hairpin clearly. All views were generated using the program Insight II (Biosym Technologies) from the Protein Data Bank file; note that this program adds a virtual bond between residues 15 and 33 in the six subunits in which the mobile loop is not resolved.

Figure 3



Surface map of the electrostatic potential of the inside of the GroES dome as calculated by the program GRASP [37]. A cutaway view of the complex, containing four of the seven GroES monomers, is shown, allowing visualization of the inside surface of GroES (seven-fold axis indicated by the arrow). Note that the inside of the GroES dome, which probably forms a continuous space with the GroEL central channel, is highly charged. The electrostatic potential is contoured in the range from $-10\ k_BT$ (red) to $+10\ k_BT$ (blue), where k_B is Boltzmann's constant and T is the absolute temperature (°K).

The chemical nature of the surface of the inner cavity of GroES is of particular interest, as it is likely to form a continuous space with the enlarged GroEL cavity in GroEL–GroES complexes, a space in which protein folding can initiate and, in some cases, perhaps proceed to completion [33]. The inside surface of GroES is very hydrophilic, both within the dome region and also at the bottom interface between the subunits (Fig. 3). The nature of the GroEL portion of the channel in a GroEL–GroES complex is still unclear. Prior to binding GroES, the inside of the GroEL central channel is apolar, but the residues forming it are subject to displacement upward and outward upon GroES binding and may no longer form the surface of the channel in the binary complex [23]. Changes in channel surface properties of GroEL after GroES binding could contribute to the different functional roles of these states. The hydrophobic nature of the GroEL channel face prior to GroES binding is probably important in stabilizing the exposed hydrophobic surfaces of the bound, unfolded protein. By contrast, a more hydrophilic surface at GroEL, coupled with that of the GroES inner cavity, might favor interaction with more native-like conformations of the polypeptide that expose relatively more hydrophilic surface.

Blowing the roof open

Hunt *et al.* point out that the β-hairpin roof structure appears to be weakly-packed, as, on average, only 133 $Å^2$ per subunit of accessible surface area is buried in the interface between adjacent hairpins [1]. In addition, crystallographic analysis of GroES shows higher-than-expected thermal motion and increased flexibility coinciding with the roof β-hairpin. The functional significance of this flexibility is not known. One possibility, raised by Hunt *et al.*, is that the roof of GroES might open out to allow escape of non-native polypeptides from the central channel and initiate folding. Recent studies, however, reveal that, in the case of rhodanese and green fluorescent protein (GFP), when GroES release is prevented, these substrate proteins are able to fold while remaining sequestered under GroES [33]. Thus, at least for some substrates, passage of non-native polypeptides through GroES is not required for productive folding.

The GroES mobile loop

An elegant NMR study several years ago [36] identified a mobile loop, residues 17–32, in free GroES, whose resonances were broadened upon binding of GroES to GroEL, suggesting that this segment became immobilized via direct interaction with GroEL. As might be expected for unbound GroES, this mobile loop region is undetermined in six of the seven subunits in the GroES crystal structure, but in the remaining subunit, it fortuitously forms a lattice contact with another GroES molecule, allowing its visualization as a β-hairpin loop structure about 15–20 Å long. Because some of the same residues in GroEL that bind GroES are also required for polypeptide binding [5], it follows that the mobile loop may be able to compete for, and possibly displace, polypeptide from the apical sites. This interaction would have some unusual features. GroES supplies in the loop only a short segment for a putative interaction with what, in the unliganded form of GroEL, amounts to a tier of three structures nearly 30 Å in height [3,5]. Because the loop could only interact with a limited extent of this face, it seems unlikely that binding of the loop would result in complete displacement of the polypeptide. It is possible, however, that allosteric changes of the apical structures might result from binding of the loop, and that such changes might cause polypeptide release.

Because ATP binding with GroES produces rapid and extensive acquisition of polypeptide flexibility and productive folding [33], it seems to promote further allosteric movement of the apical domains of the *cis* ring, releasing bound peptide into the central channel. Presumably GroES must also move in response to such apical GroEL movements, remaining associated with the *cis* ring until a further conformational change of the apical domains, driven by hydrolysis of ATP in the *trans* ring, leads to its release. It is thus possible that the GroEL–GroES chamber has two distinct conformations: a folding-inactive state, for example, in the presence of ADP, in which polypeptide is held rigidly; and a folding-active state, induced by ATP binding, in which polypeptide is released into the channel in a fully flexible form. The definition of these conformations at atomic level

will now be critical to our further understanding of the features of the GroEL–GroES folding chamber that facilitate polypeptide folding.

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